REMARKS

Claims 1 – 10, 43 – 45 and 49 are pending in the application. Claims 4 – 42 and 46 – 48 have been cancelled. Claims 1 and 49 have been amended. No new claims have been added.

Any cancellation of the claims should in no way be construed as acquiescence to any of the Examiner's rejections and was done solely to expedite the prosecution of the application. Applicant reserves the right to pursue the claims as originally filed in this or a separate application(s).

Claim Rejections Withdrawn

The Examiner has withdrawn the rejection of claims 1 – 3, 8 and 43 – 45 under 35 USC 102(b) as being anticipated by US Patent 5,608,060.

The Examiner has withdrawn the rejection of claims 1 – 3, 8 – 10, 43 – 45 and 49 under 35 USC 102(e) as being anticipated by US Patent 7,265,085.

The Examiner has withdrawn the rejection of claims 1 – 3, 8 – 10, 43 – 45 and 49 under 35 USC 102(e) as being anticipated by US Patent 7,265,085.

The Examiner has withdrawn the rejection of claims 1 and 8 - 12 under 35 USC 103 (a) over US Patent 7,265,085.

35 U.S.C. §112, first paragraph

Enablement

Claims 1 – 3, 8 – 10 and 43 – 45 were rejected under 35 U.S.C. §112, first paragraph. The Examiner argues that "the specification, while being enabling only for a method of making a

targeted glycoconjugate comprising a specific bioactive agent and a specific targeting compound wherein the bioactive agent and the targeting compound are joined by a modified UDP-galactose-Acetyl group (UDP-GalNAc) having a ketone functional group appended at the C-2 position of the galactose ring using the mutant Y289L galactose transferase for detection assays, does not reasonably provide enablement for any targeted glycoconjugate comprising any bioactive agent and any targeting compound wherein the bioactive agent and the targeting compound are joined by any modified saccharide compound for use in any medical therapy, any pharmaceutical composition comprising any targeted glycoconjugate comprising any bioactive agent and any targeting compound wherein the bioactive agent and then targeting compound are joined by any modified saccharide compound." (Office Action, p.3). Applicants respectfully disagree.

The instant claims are directed to a targeted glycoconjugate comprising a bioactive agent and a targeting compound, wherein the bioactive agent and targeting compound are joined by a modified UDP galactose acetyl group (UDP-GalNAc), and wherein the modified UDP-GalNAc comprises a ketone group attached to the C2 position of the galactose ring.

The Examiner argues that "(e)nablement is not commensurate in scope with how to make any targeted glycoconjugate comprising any bioactive agent and any targeting compound, any targeting compound is any glycoprotein wherein the bioactive agent and the targeting compound are joined by any modified saccharide compound wherein said modified saccharide compound comprises galactose and any reactive functional group, any functional group such as ketone group attached to the C2 position of the galactose ring for any and all medical therapy or diagnosis." (Office Action, p.4).

The claims have been amended to recite that **the bioactive agent and targeting compound are joined by a modified UDP galactose acetyl group (UDP-GalNAc), where the modified UDP-GalNAc comprises a ketone group attached to the C2 position of the galactose ring.** Accordingly, the specification enables any person skilled in the art to make and use the invention commensurate in scope of the present claims.

The Examiner argues that the claims encompass innumerous targeted glycoconjugate comprising any bioactive agent and any targeting compound wherein the bioactive agent and the targeting compound are joined by any modified saccharide compound. (Office Action, p.4).

Applicants point out that the specification teaches saccharide compounds of the present invention, and in particular galactose, are particularly modified at the C2 position. The specification teaches that the C2 position is favorable over other positions on the galactose ring because GalT has been shown to tolerate unnatural substrates containing minor substitutions at the C2 positions. Applicants teach that appending the ketone functionality at the C-2 position of the galactose ring. At page 48 of the specification, Applicants describe a strategy for the rapid and sensitive detection of O-GlcNAc glycosylated proteins, where experiments show that "the ketone functionality was appended at the C-2 position of the galactose ring because GalT has been shown to tolerate unnatural substrates containing minor substitutions at the C-2 positions, including 2-deoxy, 2-amino, and 2-N-acetyl substituents (Ian et al., 2001; Wong et al., 1995) (and)... 2-deoxy-Gal was transferred at rates comparable to Gal, whereas 3-, 4, and 6-deoxy-Gal were transferred at reduced rates." (page 48).

Accordingly, the claims recite that the modified UDP-GalNAc comprises a ketone group attached to the C2 position of the galactose ring. Further, the specification teaches modification of the saccharide to include a functional group, such as a ketone group, aids in the attachment of the bioactive agent, and provides examples of such attachments. For example, on page 10, the specification teaches that "the modified saccharide (S) may include a ketone moiety which can be reacted with an amino group of a bioactive agent of interest so as to form a covalent bond between the two."

The specification teaches at page 11 beginning at line 5, various methods that can be used to bind the bioactive agent to the modified saccharide:

> The methods used to bind the bioactive agent (B) to the modified saccharide (S) depend on the structure of the bioactive agent. The bioactive compounds may preferably include a functional group which may be useful, for example, in forming covalent bonds with the saccharide residue, which are not generally critical for the activity of the bioactive agent. Examples of such functional groups

include, for example, amino (--NH.sub.2), hydroxy (--OH), carboxyl (--COOH), thiol (--SH), phosphate, phosphinate, ketone group, sulfate and sulfinate groups. If the bioactive compounds do not contain a useful group, one can be added to the bioactive compound by, for example, chemical synthetic means. Where necessary and/or desired, certain moieties on the components may be protected using blocking groups, as is known in the art, see, e.g., Green & Wuts, Protective Groups in Organic Synthesis (John Wiley & Sons) (1991).

Exemplary covalent bonds by which the bioactive compounds may be associated with the saccharide residue (S) include, for example, amide (--CONH--); thioamide (--CSNH--); ether (ROR', where R and R' may be the same or different and are other than hydrogen); ester (--COO--); thioester (--COS--); --O--; --S--; --S.sub.n--, where n is greater than 1, preferably about 2 to about 8; carbamates; --NH--; --NR--, where R is alkyl, for example, alkyl of from about 1 to about 4 carbons; urethane; and substituted imidate; and combinations of two or more of these.

Covalent bonds between a bioactive agent (B) and a modified saccharide residue (S) may be achieved through the use of molecules that may act, for example, as spacers to increase the conformational and topographical flexibility of the compound. Examples of such spacers include, for example, succinic acid, 1,6-hexanedioic acid, 1,8-octanedioic acid, and the like, as well as modified amino acids, such as, for example, 6-aminohexanoic acid, 4-aminobutanoic acid, and the like.

The Examiner argues that "(t)he specification provided little or no guidance as to the binding specificity of the targeting compound beyond the mere mention of a laundry list of targeting molecules, bioactive agents joined by a list of modified saccharide compounds." (Office Action, p.4). The Examiner argues that "there is no guidance as to the binding specificity of the targeting compound for the claimed glycoconjugated (and) given the numerous unspecified glycoconjugates, there is a lack of in vivo working example of such glycoconjugate could treat any disease such as AIDS." (Office Action, p.4).

According to the MPEP at 2164.02, "compliance with the enablement requirement of 35 U.S.C. 112, first paragraph, does not turn on whether an example is disclosed." Moreover,

Applicant does not need to demonstrate therapeutic effects for particular diseases to enable the invention as claimed.

The invention as claimed is a targeted glycoconjugate comprising a bioactive agent and a targeting compound, wherein the bioactive agent and targeting compound are joined by a modified UDP galactose acetyl group (UDP-GalNAc), and wherein the modified UDP-GalNAc comprises a ketone group attached to the C2 position of the galactose ring.

The present invention features glycoconjugates in which a bioactive agent is bound through a modified saccharide residue, e.g., UDP-GalNAc, to a compound which has an affinity for a target cell.

In the Examples, Applicants describe the rapid and sensitive detection of O-GlcNAc glycoslated proteins. As described by Applicants at page 48 of the specification, "the approach capitalizes on the substrate tolerance of GalT, which allows for chemoselective installation of an unnatural ketone functionality to O-GlcNAc modified proteins. The ketone moiety has been well-characterized in cellular systems as a neutral, yet versatile, chemical handle (Cornish et al., 1996; Mahal et al., 1997; Datta et al., 2002). Here, it serves as a unique marker to "tag" O-GlcNAc glycosylated proteins with biotin. Once biotinylated, the glycoconjugates can be readily detected by chemiluminescence using streptavidin conjugated to horseradish peroxidase (HRP)." (line 14 – 21).

Applicants demonstrate in the Examples the ability of GalT to label the peptide TAPTS(O-GlcNAc)TIAPG, which encompasses an O-GlcNAc modification site within the protein CREB. Applicants use wild-type GalT and show that only partial transfer of the keto-sugar was observed by LC-MS, however when the Y289L mutant was used there was greater activity and complete conversion. (see page 40, line 14 – 22). Further, Applicants show that the same strategy can be used for the labeling of the O-GlcNAc glycosylated protein CREB (see, e.g. page 45, line 8 – 23).

As described in the specification at page 10, line 15, "the targeting compound (T)…is covalently bonded to a saccharide residue (S) with the use of a galactosyltranserfase enzyme, preferably beta-1,4-galactosyltransferase (GalT). In one embodiment of the invention, a modified saccharide (S) is covalently associated with the targeting compound with the use of a genetically engineered GalT, such as Y289L GalT (as discussed above). **The targeting compound can be any**

naturally occurring glycoprotein, glycolipid or carbohydrate or can be engineered, through chemical or recombinant techniques. For example, if the targeting compound does not include a GlcNAc residue, the compound can be engineered, either through recombinant or chemical techniques known in the art, so as to include such a residue. Preferably, the targeting compound includes an N-acetylglucosamine (GlcNAc) residue."

The specification teaches a wide variety of bioactive agents that may be used, and that are known in the art as useful in therapeutic or diagnostic methods or in medical therapies. For example beginning at page 12, line 8, the specification teaches:

> A wide variety of bioactive agents (B) may be included in the compounds of the present invention, such as any biologically active, therapeutic or diagnostic compound/composition. In general, the term bioactive agent includes, but is not limited to: polypeptides, including proteins and peptides (e.g., insulin); releasing factors and releasing factor inhibitors, including Luteinizing Hormone Releasing Hormone (LHRH) and gonadotropin releasing hormone (GnRH) inhibitors; carbohydrates (e.g., heparin); nucleic acids; vaccines; and pharmacologically active agents such as anti-infectives such as antibiotics and antiviral agents; anti-fungal agents; analgesics and analgesic combinations; anesthetics; anorexics; anti-helminthics; anti-arthritic agents; respiratory drugs, including anti-asthmatic agents and drugs for preventing reactive airway disease; anticonvulsants; antidepressants; anti-diabetic agents; anti-diarrheals; anticonvulsants; antihistamines; anti-inflammatory agents; toxins, anti-migraine preparations; anti-nauseants; anticancer agents, including anti-neoplastic drugs; anti-parkinsonism drugs; anti-pruritics; anti-psychotics; antipyretics; antispasmodics; anticholinergics; sympathomimetics; xanthine derivatives; cardiovascular preparations including potassium and calcium channel blockers, beta-blockers, alpha-blockers, cardioprotective agents; anti-arrhythmics; anti-hyperlipidemic agents; anti-hypertensives; diuretics; anti-diuretics; receptor agonists, antagonists, and/or mixed function agonist/antagonists; vasodilators including general coronary, peripheral and cerebral; central nervous system stimulants; vasoconstrictors; cough and cold preparations, including decongestants; enzyme inhibitors; hormones such as estradiol, testosterone, progesterone and other

steroids and derivatives and analogs, including corticosteroids; hypnotics; hormonolytics; immunosuppressive agents; muscle relaxants; parasympatholytics; central nervous system stimulants; diuretics; hypnoticsleukotriene inhibitors; mitotic inhibitors; muscle relaxants; genetic material, including nucleic acid, RNA, DNA, recombinant RNA, recombinant DNA, antisense RNA, antisense DNA, hammerhead RNA, a ribozyme, a hammerhead ribozyme, an antigene nucleic acid, a ribo-oligonucleotide, a deoxyribonucleotide, an antisense ribo-oligonucleotide, and/or an antisense deoxyribo-oligonucleotide; psychostimulants; sedatives; anabolic agents; vitamins; herbal remedies; anti-metabolic agents; anxiolytics; attention deficit disorder (ADD) and attention deficit hyperactivity disorder (ADHD) drugs; neuroleptics; and tranquilizers.

Beginning at page 24, the specification discusses therapeutic uses. Accordingly, demonstration of specific therapeutic effects for particular diseases to enable the invention as claimed is not necessary.

Applicants have further exemplified that antibodies can be galactosylated with Y289L GalT having a chemical handle at the C2 position in Bioconjugate Chem. 2009, 20, 1228 – 1236 (provided herein). Applicants describe the utility of Y289L GalT to transfer a sugar residue with C2-keto-Gal (or GalNAz) from their UDP derivatives to the N-acetylglucosamine residue of glycoproteins or glycopeptides. (see, e.g. Figure 5 on page 1233). Moreover, Applicants teach that the conjugation technology is a viable method that can be used for detection and targeting therapies. (see, p.1229). In Bioconjugate Chem. 1009, 20, 1383- 1389 (provided herein), Applicants describe the biological activity of the described glycoconjugates. For example, Applicants describe C-terminal extended fusion polypeptides of recombinant scFv fusion proteins that are used as the acceptor substrate for human polypeptide-alpha-N-acetylgalactosaminyltransferase II that transfers either GalNAc or 2-keto-Gal from their respective UDP-sugars to the side-chain hydroxyl group of the Thr residue(s). The fusion scFv proteins with the modified galactose are then conjugated with a fluorescence probe, Alexa488, that carries an orthogonal reactive group. The fluorescence labeled scFv proteins bind specifically to a human breast cancer cell line (SK-BR-3) that overexpresses the HER2 receptor, indicating that the in vitro folded scFv fusion proteins are biologically active and the presence of

conjugated multiple Alexa488 probes in their C-terminal end does not interfere with their binding to the antigen.

Taken together, the teachings of the specification and knowledge of one of skill in the art enables one of skill in the art to practice the full scope of the claimed invention without having to resort to undue experimentation. Applicants accordingly request that the rejection be reconsidered and withdrawn.

Written Description

Claims 1 – 3, 8 – 12 and 43 – 45 were rejected under 35 U.S.C. §112, first paragraph, for allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor, at the time the application was filed, had possession of the claimed invention. (Office Action, p.8). Applicants respectfully disagree.

The Examiner argues that "claims 1, 9 and 45 are broadly drawn to any targeted glycoconjugate comprising any bioactive agent and any targeting compound wherein the bioactive agent and the targeting compound are joined by any modified saccharide compound for use in any medical therapy." (Office Action, p.8). Applicants respectfully disagree.

The Examiner argues that "claim 2 is broadly drawn to any targeted glycoconjugate comprising any bioactive agent such as any and all polypeptide, any and all releasing factor, any and all releasing factor inhibitor, any and all carbohydrate, any and all nucleic acid and any and any targeting compound wherein the bioactive agent and the targeting compound are joined by any modified saccharide compound wherein the modified saccharide compound comprises galactose and any reactive functional group attached to the C2 position of the galactose ring for use in any medical therapy." (Office Action, p.8). Applicants respectfully disagree.

The Examiner argues that "claim 3 is broadly drawn to any targeted glycoconjugate comprising any bioactive agent and any targeting compound such as any glycoprotein wherein the bioactive agent and the targeting compound are joined by any modified saccharide compound." (Office Action, p.8 - 9). Applicants respectfully disagree.

The Examiner argues that "claim 8 is broadly drawn to any targeted glycoconjugate comprising any bioactive agent and any targeting compound wherein the bioactive agent and the targeting compound are joined by any modified saccharide compound such as modified galactose." (Office Action, p.0). Applicants respectfully disagree. Claim 8 has been cancelled. Applicants respectfully request that this rejection be withdrawn.

The Examiner argues that "claim 10 is broadly drawn to any targeted glycoconjugate comprising any bioactive agent and any targeting compound wherein the bioactive agent and the targeting compound are joined by any modified galactose further comprises any reactive functional group." (Office Action, p.9). Applicants respectfully disagree. Claim 10 has been cancelled. Applicants respectfully request that this rejection be withdrawn.

The Examiner argues that "claim 43 is broadly drawn to any and all pharmaceutical composition comprising any and all targeted glycoconjugate comprising any bioactive agent and any targeting compound wherein the bioactive agent and the targeting compound are joined by any modified saccharide compound and a pharmaceutically acceptable carrier." (Office Action, p.9). Applicants respectfully disagree.

The Examiner argues that "claim 44 is broadly drawn to a kit comprising any targeted glycoconjugate comprising any bioactive agent and any targeting compound wherein the bioactive agent and the targeting compound are joined by any modified saccharide compound and a pharmaceutically acceptable carrier." (Office Action, p.9). Applicants respectfully disagree.

In the interest of compact prosecution, the above rejections will be addressed together.

The Examiner argues that "the scope of each genus includes many members with widely differing structural, chemical, and physiochemical properties of targeting compound and bioactive agent such as widely differing amino acid sequences, nucleotide sequences and biological functions in the claimed glycoconjugate." (Office Action, p.10). The Examiner argues further that "each genus is highly variable because a significant number of structural and biological differences between genus members exist." (Office Action, p.10). Applicants disagree.

As amended, the claims are sufficiently described in the specification.

The claims have been amended to particularly recite targeted glycoconjugate compounds comprising a bioactive agent and a targeting compound, wherein the bioactive agent and targeting compound are joined by a modified UDP galactose acetyl group (UDP-GalNAc), and wherein the modified UDP-GalNAc comprises a ketone group attached to the C2 position of the galactose ring.

As discussed above, the claims have been amended to recite that **the bioactive agent and targeting compound are joined by a modified UDP galactose acetyl group (UDP-GalNAc), where the modified UDP-GalNAc comprises a ketone group attached to the C2 position of the galactose ring**.

The specification teaches that saccharide compounds of the present invention, and in particular galactose, are particularly modified at the C2 position. The specification teaches that the C2 position is favorable over other positions on the galactose ring because GalT has been shown to tolerate unnatural substrates containing minor substitutions at the C2 positions. Applicants describe a strategy for the rapid and sensitive detection of O-GlcNAc glycoslated proteins, where experiments show that "the ketone functionality was appended at the C-2 position of the galactose ring because GalT has been shown to tolerate unnatural substrates containing minor substitutions at the C-2 positions, including 2-deoxy, 2-amino, and 2-N-acetyl substituents (Ian et al., 2001; Wong et al., 1995) (and)... 2-deoxy-Gal was transferred at rates comparable to Gal, whereas 3-, 4, and 6-deoxy-Gal were transferred at reduced rates." (page 48).

Modified saccharide compounds are described at page 9.

Targeting compounds are described at page 10 and page 18. For example, antibodies are given as an example of a targeting compound at page 20.

Bioactive agents are described beginning at page 10.

Applicants submit that the claims are sufficiently described in the specification to reasonably convey to one skilled in the relevant art that the inventor, at the time the application was filed, had possession of the claimed invention. Applicants respectfully request that the foregoing rejections be withdrawn.

35 U.S.C. §103(a)

Claims 1 – 3, 8 – 10, 43 – 45 and 49 stand rejected under 35 U.S.C. §103(a) over US Patent No. 7,265,085 (the '085 reference herein) and in view of Ramakrishnan et al. (J Biol Chem 277 (23):20833 – 20839, June 2002) and Hang et al. (J Am Chem 123: 1242 – 1243, 2001). Applicants respectfully traverse the rejection.

The claims have been set forth above.

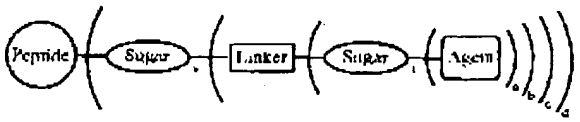
The Examiner argues that "(t)he '085 reference teaches various targeted glycoprotein such as transferring-SA linker-GDNF wherein the reference targeting compound such as transferrin and bioactive agent such as GDNF are joined by a modified saccharide compound such as o-Glc-NAc modified galactose using beta-1,4 galactosyl transferase." (Office Action, p.14). The Examiner argues that

The '085 reference is directed to methods of remodeling a peptide to attach a specific glycan structure. The '085 reference teaches that at least one of the glycosyl donors comprises a modifying group (and) preferably, the modifying group is a member selected from the group consisting of a polymer, a therapeutic moiety, a detectable label, a reactive linker group, a targeting moiety and a peptide. That is, the '085 reference teaches that the glycans structures are remodeled in order to be useful (see col. 63 – 65). Conjugates of the invention are described beginning at col. 66:

> In a first aspect, the present invention provides a conjugate between a peptide and a selected moiety. **The link between the peptide and the selected moiety includes an intact glycosyl linking group interposed between the peptide and the selected moiety**. As discussed herein, the selected moiety is essentially any species that can be attached to a saccharide unit, resulting in a "modified sugar" that is recognized by an appropriate transferase enzyme, which appends the modified sugar onto the peptide. (col. 66 – 67, emphasis added.

Typical conjugates of the invention are shown by the structure at col. 67, line 5, where "symbols a, b, c, d and s represent a positive, non-zero integer; and t is either 0 or a positive integer. The 'agent' is a therapeutic agent, a bioactive agent, a detectable label, water-soluble

moiety or the like...(and) (t)he linker can be any of a wide array of linking groups, infra...(or) a single bond or a "zero order linker."



The '085 reference exemplifies such a conjugate at col. 68, line 6, where "EPO is conjugated to transferrin...(or) EPO is conjugated to glial derived neurotropic growth factor (GDNF). In these embodiments, each conjugation is accomplished via **a bifunctional linker that includes an intact glycosyl linking group at each terminus of the PEG moiety**, as aforementioned." (emphasis added).

Clearly, the glycoconjugates that are taught by the '085 reference are different from the present claims, where the bioactive agent and targeting compound are joined by a modified UDP galactose acetyl group (UDP-GalNAc), where the modified UDP-GalNAc comprises a ketone group attached to the C2 position of the galactose ring. The structure of these conjugates is different from the present invention as claimed.

None of the Ramakrishnan or Hang references cure the defects of the '085 reference.

The Examiner argues that "Ramakrishnan et al. teach a modified beta-1,4 galactosyltransferase having a tyrosine at position 289 substitute for Lysine that enhances the GalNAc-transferase activity equal to that of Gal-T activity." (Office Action, p.15).

The Examiner argues that "Hang et al. teach the use of unnatural or modified monosaccharide such as 2-ketosugars or 2-keto isotere of GalNAc sugar or 2-acetaminodsugars as the substrate for GalNAc transferase for metabolic glycoprotein engineering in CHO cells. Hang et al. further teach the ketone reactive group produced by 2-ketosugars can be used as a molecular handle and more accessible for chemical reaction with biotin hydrazide." (Office Action, p.15).

The Ramakrishnan and Hang references do not make up for the defects of the '085 reference. The '085 reference does not teach a targeted glycoconjugate comprising a bioactive agent and targeting compound that are joined by a modified UDP galactose acetyl group (UDP-GalNAc). Nor

does the '085 reference provide teaching or suggestion to modify any position of the saccharide ring preferably over any other position. It would not have been obvious to one of ordinary skill in the art at the time the invention was made to substitute the beta-1,4 galactosyl transferase that catalyse the transfer of galactose in the target conjugate of the '085 patent for the modified beta-1,4 galactosyltransferase taught by the Ramakrishnan reference using any modified monosaccharide such as 2-ketosugars or 2-ketoisostere of GalNAc as a molecular handle as taught by the Hang reference.

In view thereof, reconsideration and withdrawal of the rejection are requested.

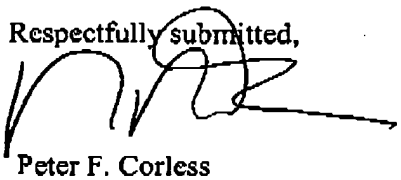
CONCLUSION

For the reasons provided, Applicant submits that all claims are allowable as written and respectfully requests early favorable action by the Examiner.

If the Examiner believes that a telephone conversation with Applicant's attorney/agent would expedite prosecution of this application, the Examiner is cordially invited to call the undersigned attorney of record.

Date: September 8, 2009

Respectfully submitted,



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Site Specific Conjugation of Fluoroprobes to the Remodeled Fc N-Glycans of Monoclonal Antibodies Using Mutant Glycosyltransferases: Application for Cell Surface Antigen Detection

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The Fc N-glycan chains of four therapeutic monoclonal antibodies (mAbs), namely, Avastin, Rituxan, Remicade, and Herceptin, released by PNGase F, show by MALDI analysis that these biantennary N-glycans are a mixture of G0, G1, and G2 glycoforms. The G0 glycoform has no galactose on the terminal GlcNAc residues, and the G1 and G2 glycoforms have one or two terminal galactose residues, respectively, while no N-glycan with terminal sialic acid residue is observed. We show here that under native conditions we can convert the N-glycans of these mAbs to a homogeneous population of G0 glycoform using β1,4 galactosidase from *Streptococcus pneumoniae*. The G0 glycoforms of mAbs can be galactosylated with a modified galactose having a chemical handle at the C2 position, such as ketone or azide, using a mutant β1,4-galactosyltransferase (β1,4Gal-T1-Y289L). The addition of the modified galactose at a specific glycan residue of a mAb permits the coupling of a biomolecule that carries an orthogonal reactive group. The linking of a biotinylated or a fluorescent dye carrying derivatives selectively occurs with the modified galactose, C2-keto-Gal, at the heavy chain of these mAbs, without altering their antigen binding activities, as shown by indirect enzyme linked immunosorbent assay (ELISA) and fluorescence activated cell sorting (FACS) methods. Our results demonstrate that the linking of cargo molecules to mAbs via glycans could prove to be an invaluable tool for potential drug targeting by immunotherapeutic methods.

INTRODUCTION

Monoclonal antibodies are increasingly becoming important therapeutic agents to target drugs to the tissue in need of treatment, leaving the normal tissue unharmed (*1*). They are commonly used in combination with chemotherapeutic agents for cancer treatment (*2*). The concept of making of antibody−drug conjugates (ADC) by which the antibody is used as a vehicle to deliver a cargo molecule to the tumor cell is gaining immense interest (*3*−*5*). Generally, the ADCs are antibodies conjugated to cytotoxic substances, such as drugs, toxins, and radioisotopes. The conjugated antibody recognizes and binds to cell surface antigen(s). At this point, the cargo molecules must be internalized into the target cells and released from the antibodies for activation of the drug.

All licensed therapeutic antibodies are of the IgG class; these are complex glycoproteins with two main functions, including a multivalent antigen binding through the Fab variable domains and effector functions through the Fc constant domain. IgG molecules are N-glycosylated in the CH_2 domain of the Fc fragment at the conserved Asn 297. In human IgG1, which is the main subtype used for therapeutics, the majority of the Fc glycans are complex biantennary structures with variable galactosylation: 0, 1, or 2 terminal galactoses corresponding to G0, G1, and G2 glycoforms, respectively, and <10−14% are sialylated (*6*, *7*). Only 15−20% of human IgG1 are also glycosylated in the Fab region of the molecule (*7*, *8*). Recent in vitro studies have established the importance of the Fc glycosylation profile of the IgG molecule on its effector functions: complement-dependent, cell-mediated cytotoxicity (CDC) and antibody-dependent, cell-mediated cytotoxicity (ADCC), such that removal of terminal galactose from the IgG can reduce the CDC but not affect ADCC activity (*9*). However, the use of rat hybridoma YB2/0 cells has permitted the production of mAbs that lack fucose in the conserved core oligosaccharide increasing the ADCC activity of the IgG, allowing the antibody to be effective at lower doses (*10*). Thus, glycosylation of recombinant monoclonal antibodies has been the focus of attention of the pharmaceutical industry to produce a homogeneous human-type glycosylation product, irrespective of the system/means by which the antibodies are produced.

After years of work to produce successful monoclonal antibodies as targeted drug therapeutics, researchers have developed 6 unconjugated and 3 antibody−drug conjugates that are currently approved for cancer treatment (*11*). Generally, the labeling of antibodies with the cargo molecules involves chemical methods which are difficult to control because the chemical reactions occur at tyrosine, lysine, aspartic, and glutamic acid residues that are distributed randomly on the surface of the protein. The result is the production of proteins with compromised activities (*12*). In order to maintain the biological activity of the protein and to optimize the production of homogeneous proteins, several methods have been developed for site-specific modification of the carriers. In the monoclonal antibodies, this site should be selected such that it is away from the antigen binding site. The oligosaccharide moieties of

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immunoglobulins are not involved in the process of antigen binding and are N-linked to Asn297 on the heavy chain in the Fc domain of the immunoglobulin far from the antigen binding site. The conjugation of monoclonal antibodies to the cargo molecules via the oligosaccharides has been carried out by chemical or enzymatic methods, resulting in the formation of aldehydes (*13, 14*). Generally, the chemistry used for the modification of the sugar moieties present on glycoproteins such as the immunoglobulin's is mild periodate oxidation of sialic acid residues, resulting in the generation of aldehyde groups, which can then be used for coupling (*15, 16*). However, less than 10−14% of mAbs are sialylated (*6, 7*). An alternative method for the generation of aldehyde groups on the oligosaccharide moieties of glycoproteins is the use of enzymes (*16−18*). However, the maximum number of aldehyde groups is dictated by the structure/linkage of the sugar; thus, the heterogeneous nature of the sugar moieties on the monoclonal antibodies can result in poor conjugation.

Recently, new chemoenzymatic methods for conjugation have been developed for site-specific conjugation (*19−21*). We have shown the utility of the galactosyltransferase mutant to transfer a sugar residue with a chemically reactive functional group (e.g., C2-keto-Gal or GalNAz) from their UDP derivatives to the N-acetylglucosamine residue of glycoproteins or glycopeptides (*20, 21*). Using this method, we have shown that the N-glycan moiety of the IgG molecule can be used as the substrate for the transfer of C2-keto-Gal sugar by the mutant β1,4Gal-T1-Y289L (*20*). After the transfer of the modified sugar residue, the chemical handle is used for selective conjugation with a biomolecule that has an orthogonal reactive group, such as an aminooxy linked to biotin (*19−21*).

Here, we show that one can take advantage of the unique heterogeneous oligosaccharide linked at Asn297 in the mAbs that can be made homogeneous by a simple enzymatic procedure and obtain a population of mAbs having a set of glycans of the G0 glycoform that are subsequently fully galactosylated to a G2 glycoform, with the modified C2-keto-Gal as monitored by MALDI-TOF analysis of the PNGase F-treated samples. The transferred C2-keto-Gal is coupled to an aminooxy-biotin, which was previously detected by chemiluminescence techniques (*20, 22*) and here by MALDI-TOF analysis of the N-glycans. Using ELISA methodology, we show that the coupling of aminooxy-biotin to C2-keto-Gal does not compromise the antigen (Ag) binding site. Furthermore, Herceptin with C2-keto-Gal modified N-glycans coupled to Alexa Fluor 488 C_5-aminooxyacetamide binds to HER2-receptor-expressing cells, as shown by FACS methodology. Therefore, our conjugation technology presents a viable method to quantify the cargo molecules to be used for detection and targeting therapies.

EXPERIMENTAL PROCEDURES

REAGENTS. Rituxan (Rituximab) and Remicade (Infliximab) are recombinant chimeric monoclonal antibodies (mAbs), and Avastin (Bevacizumab) and Herceptin (Trastuzumab) are recombinant humanized mAbs (Genentech, Inc., South San Francisco, CA), purchased via the NIH pharmacy (Bethesda, MD). FITC-conjugated goat antihuman IgG (whole molecule) was from Sigma-Aldrich (St. Louis, MO). Alexa Fluor 488 C_5-aminooxyacetamide, bis (triethylammonium) salt (Alexa Fluor 488 hydroxylamine) was from Invitrogen (Eugene, OR). Recombinant β1,4-galactosidase from *Streptococcus pneumoniae* was from Calbiochem (San Diego, CA). Peptide *N*-glycoside F (PNGase F) was from New England Biolabs (Ipswich, MA). Microcon Ultracel YM-50 centrifugation devices came from Millipore Corporation, (Bedford, MA). Protein A-Sepharose 4B conjugate was from Invitrogen (Eugene, OR). 2,5-Dihydrobenzoic acid (DHB) came from Sigma-Aldrich (St. Louis, MO). Micro biospin chromatography columns P-30 were from Bio-Rad (Hercules, CA). Recombinant human vascular endothelial growth factor (VEGF) was from Thermo Scientific and *p*-nitrophenyl phosphate from Sigma-Aldrich (St. Louis, MO). UDP-C2-keto-Gal was synthesized as described previously (*19*).

β 1,4 Galactosyltransferase Expression in *E. coli* and in Vitro Folding of Inclusion Bodies. The enzymes β1,4Gal-T1[1] and β1,4Gal-T1-Y289L used in this study have been previously described (*23, 24*). Inclusion bodies were purified from the bacterial pellet as described earlier (*23, 24*). The in vitro folding of the enzymes was carried out in a way similar to that of β1,4Gal-T1 (*23*), with a few modifications. Typically, 100 mg of sulfonated protein were folded for 48 h in 1 L of folding solution that contained oxido-shuffling reagents and 550 mM arginine-HCL (*23*). The presence of arginine in the folding solution enhances the folding efficiency of β1,4Gal-T1-Y289L.

Degalactosylation of Monoclonal Antibodies. Avastin, Rituxan, Remicade, or Herceptin were washed with 50 mM sodium phosphate pH 6.0, using a Microcon Ultracel YM-50 centrifugation device. The samples at 8 mg mL^{-1} were incubated with 100 mU of recombinant *Streptococcus pneumoniae* β1,4-galactosidase for 24 h at 37 °C. Removal of terminal galactose residues was confirmed by analysis of the N-glycans released after PNGAse F treatment by MALDI TOF spectrometry. Approximately 3 μg of mAbs was incubated in the presence or absence of PNGase F (2500 units), 16 h at 37 °C in 10 μL of G7 buffer. Samples were then purified on microspin charcoal columns (Harvard Apparatus, MA). Samples were eluted with 30% acetonitrile and analyzed by mass spectrometry. Degalactosylated monoclonal antibodies were then purified by protein A affinity chromatography.

Protein A Affinity Chromatography of mAbs. Degalactosylated samples were diluted 1:1 with 1 × PBS, pH 7.4 (binding buffer), and then added to the protein A columns (Invitrogen). The columns were washed several times with binding buffer and the mAbs were eluted with 100 mM glycine-HCL, pH 2.7. The eluted mAbs were neutralized with 1 M Tris−HCl buffer pH 8.0, concentrated, and washed with 1 × PBS, pH 7.4, using the Microcon Ultracel YM-50 centrifugation device. Protein amounts were determined using the Bio-Rad protein assay kit based on the method of Bradford (BIO-RAD), and the purity of all mAbs further verified by SDS-PAGE electrophoresis.

Transfer of C-2 keto Galactose from Its UDP-Derivative to Free GlcNAc Residues on mAbs using the Mutant β1,4Gal-T1-Y289L and Biotinylation of the mAbs. Monoclonal antibodies (12 μg) were incubated with 2 mM UDP-C2 keto-Gal and 12 μg of the mutant β1,4Gal-T1-Y289L in a 25 μL final incubation mixture containing 10 mM $MnCl_2$ and 25 mM Tris−HCl (pH 8.0). Reactions were incubated at 30 °C for 12 h. The ketone-labeled proteins were subsequently diluted to 30 μL in a mixture containing 50 mM NaOAc (pH 3.9) and 3 mM *N*-aminooxy-methylcarbonylhydrazino-D-biotin (AOB), purchased from Dojindo Laboratories. The biotinylation reactions were incubated with gentle shaking for 12−16 h at 25

[1]Abbreviations: β1,4-Gal-T1, β1,4-galactosyltransferase; ELISA, enzyme linked immunosorbent assay; FACS, fluorescence activated cell sorting; PNGase F, peptide N-glycosidase F; DHB, 2,5-dihydrobenzoic acid; DMA, *N,N*-dimethylaniline; Gal, galactose; GlcNAc, *N*-acetylglucosamine; *N*-acetyl-azido-galactosamine (Gal-2-NHCO-CH_2-N_3), GalNAz; UDP-Gal, uridine 5′-diphosphogalactose; UDP-2-keto-Gal, Uridine 5′-diphospho-2-acetonyl-2-deoxy-galactose; UDP-GalNAc, uridine 5′-diphospho-*N*-acetylgalactosamine; UDP-GalNAz, uridine 5′-diphospho-*N*-acetyl-azido-galactosamine; MALDI, matrix-assisted laser desorption ionization; MS, mass spectra; AOB, *N*-aminooxy-methylcarbonylhydrazino-D-biotin; HRP, horseradish peroxidase; FBS, fetal bovine serum; BSA, bovine serum albumin; PBS, phosphate buffer saline.

°C. A portion of the sample was treated with PNGase F for the MALDI-TOF analysis of the glycan chain, and a portion was boiled in Tris-Glycine-SDS sample buffer containing β-mercaptoethanol and analyzed by Western blot analysis as described (*19*−*21*).

SDS-PAGE Analysis of mAbs and Western Blotting of IgGs. The SDS-PAGE analyses of native or biotinylated mAbs, respectively, before and after PNGase F treatment, were performed in 14% Tris−glycine gels (Invitrogen). For Western blotting analysis, the proteins from the gels were then transferred by electrophoresis to nitrocellulose paper (0.45 μm pore size) for 2 h at 25 V and biotinylated protein bands identified as described previously (*20*).

Fluorescent Labeling of Herceptin. Fluorescent labeling reactions were carried out by treating 10 μg of C2-keto-Gal labeled Herceptin with 400 μM of Alexa Fluor 488 C_5-aminooxyacetamide in 27 μL containing 166 mM NaOAc (pH 4.9). The reactions were incubated for 2−6 h at 37 °C. The unreacted fluorescent dye was removed by purification on micro biospin chromatography columns P-30 from Bio-Rad (Hercules, CA), as per manufacturer's directions. Purified samples were separated by SDS-PAGE and fluorescence was detected using a multi view FMBIO II scanner (Hitachi).

Indirect ELISA. The binding activity of Avastin to recombinant human vascular endothelial growth factor (VEGF) was determined in a solid-phase binding assay using soluble Avastin and coated VEGF. Binding of antibodies was then detected by a specific secondary antibody in an enzyme-linked immunosorbent assay. Briefly, flat-bottom 96-well microtiter plates (Costar 3690, Corning Inc., NY) were coated with the appropriate amounts of VEGF overnight at 4 °C and were washed three times with phosphate buffer saline (PBS) pH 7.4 containing 0.05% Tween-20 and 0.1% sodium azide. The plates were blocked with 3% bovine serum albumin (BSA) in PBS with 0.05% Tween-20 for 1 h at room temperature. A serial dilution of native or modified Avastin was added to the VEGF-coated plate and incubated for 1 h at room temperature. The detection antibody conjugate (antihuman-Fc-phosphatase) (Thermo Scientific) was then applied for 1 h at room temperature. The detection of alkaline phosphatase was performed with *p*-nitrophenyl phosphate (Sigma). Samples were measured at 405 nm with a plate reader.

Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry. Typically 1−2 μL of sample was mixed with an equal volume of 2,5-dihydrobenzoic acid (DHB) matrix solution prepared by dissolving 100 mg of DHB (Sigma Chemicals) in 1 mL of a 1:1 solution of water and acetonitrile. In some instances, a matrix solution of a mixture of DHB/DMA (*N,N*-dimethylaniline) was used, which was prepared by adding 30 μL of distilled DMA to the DHB matrix (*25*). Mixtures were spotted onto a stainless steel plate after mixing the samples and matrix solutions (1 μL each). The samples were dried by evaporation at room temperature. A hybrid triple-quad time-of-flight (QqTOF) mass spectrometer (QSTAR XL, Applied Biosystems, Inc., Framingham, MA) was configured for matrix-assisted laser desorption/ionization (MALDI). Mass spectrometry (MS) data was obtained using a laser intensity = 21 000 μj, pulse rate = 20 Hz, collision gas (CAD) = 3 (high purity argon, Airgas, Inc., Frederick, MD), focusing potential (FP) = 35 V, declustering potential (DP) = 0 V, declustering potential 2 (DP2) = 20 V, and ion energy (IE1) = 0.8 V. All samples were analyzed in the positive mode with a 3 min accumulation time over an *m/z* range of 150−3000 amu.

Cell Surface Immunostaining of HER2 Receptor by FACS Analysis. To determine if the modifications in Herceptin influenced its ability to bind to the cellular HER2 receptor, we used either indirect immunostaining (when using modified Herceptin) or direct immunostaining of cells when using Alexa-conjugated-Herceptin. HER2 receptor expressing human breast adenocarcinoma cells (SKBR-3) and HER2 receptor negative human breast adenocarcinoma cells (MDA-MB-468) were purchased from the American type Culture Collection (ATCC, Manassas, VA). The cells were cultured in DMEM/F12 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and penicillin/streptomycin as antibiotics at 37 °C in a humidified atmosphere containing 5% CO_2. Other culture reagents were bought from Invitrogen (Carlsbad, CA). For immunostaining experiments, cells were harvested using a PBS-based, enzyme-free cell dissociation buffer, and suspended to a concentration of 10^7 cells per mL in PBS containing 5% FBS. The cells were further incubated for 15 min at room temperature, centrifuged and washed twice with cold PBS supplemented with 0.1% BSA (PBS−BSA), and resuspended in PBS−FBS at a concentration of 10^7 per mL. For incubation with antibodies, 100 μL cell suspension (containing 10^6 cells) were placed in 1.5 mL Eppendorf tubes. Native Herceptin, modified Herceptin, or Alexa-conjugated Herceptin (4 μg each) was added to individual samples, and incubations were continued for 1 h at 4 °C in the dark with constant shaking. At the end of incubations, samples were washed twice with cold PBS−BSA, and resuspended in 100 μL PBS−FBS. The samples incubated with Alexa-conjugated Herceptin were analyzed by FACS. The cells incubated with native Herceptin or modified Herceptin were incubated with a 1:32 dilution of FITC-conjugated goat antihuman IgG for 1 h at 4 °C in the dark with constant shaking. Cells were washed twice with PBS−BSA, and resuspended in PBS−BSA (1 mL per sample). Control samples were incubated without any primary antibody, with only the FITC-conjugated goat antihuman IgG. Cells were analyzed using a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA). Each sample was analyzed for 10 000 counts of viable cells, and all samples were run at least in duplicate.

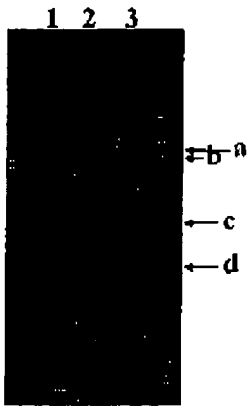

Figure 1. SDS-PAGE analysis of monoclonal antibody before and after PNGase F treatment. (1) Kaleidoscope prestained standards; (2) the native IgG; (3) IgG after treatment with PNGase F. Arrows show from top to bottom: (a) heavy chain of native IgG, (b) deglycosylated heavy chain of IgG, (c) PNGase F used for deglycosylation, and (d) light chain of IgG.

RESULTS

Glycoforms of Therapeutic Monoclonal Antibodies; MS Analysis of Native and β-galactosidase-treated IgGs. The human IgGs are N-glycosylated in the CH_2 domain of the Fc fragment and only ~15−20% are also glycosylated in the Fab domain (*7*, *8*). SDS-PAGE analysis of mAbs, Avastin, Rituxan, Remicade, and Herceptin, before and after the PNGase F treatment, shows the mobility difference only in the heavy chain

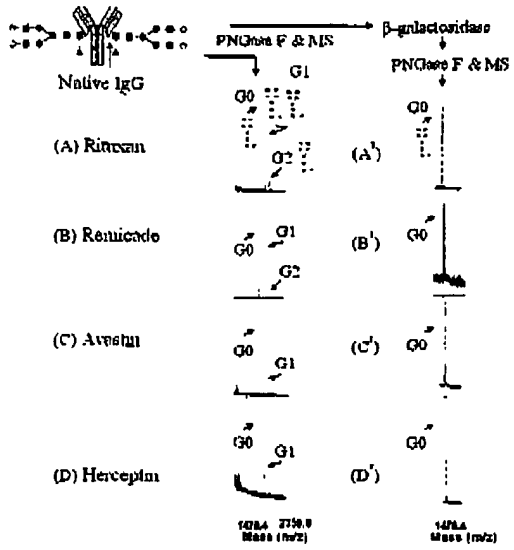

Figure 2. Glycoforms of therapeutic monoclonal antibodies; MS analysis of N-glycans after β-galactosidase treatment of IgG's. (A−D) MALDI-TOF MS analysis of N-glycans released by PNGase F treatment (red arrow shows the cleavage site) of native Rituxan (A), Remicade (B), Avastin (C), and Herceptin (D). G0 glycoform with a peak at 1485.6 *m/z*, the G1 glycoform with a peak at 1647.6 *m/z*, and G2 glycoform with a peak at 1809.6 *m/z*. mAbs were treated with β-galactosidase to obtain a homogeneous G0 glycoform population (A′−D′) MALDI-TOF analysis of N-glycans released by PNGase F treatment of β-galactosidase treated Rituxan (A′), Remicade (B′), Avastin (C′), and Herceptin (D′), respectively. Only the G0 glycoform with a peak at 1485.6 *m/z* is seen after treatment. Major peaks are annotated with the carbohydrate structure shown in the symbols for monosaccharides, according to the nomenclature adopted by the consortium for functional glycomics: GlcNAc (blue squares), mannose (green spheres), galactose (yellow spheres), and fucose (red triangles). The symbols were drawn using the GlycoWorkbench program found in the Eurocarb database http://www. Eurocarb.org/.

protein band, and not in the light chain, indicating that the oligosaccharide chain is released only from the IgG heavy chain, and not from the light chain (Figure 1).

The degree of heterogeneity of the oligosaccharides in the native mAbs was determined by carrying out the MALDI-TOF analysis of the oligosaccharide released after PNGase F treatment of mAbs. MS analysis of oligosaccharides released after PNGase F treatment of native forms of Rituxan and Remicade show that they carry oligosaccharides of G0, G1, and G2 glycoforms (Figure 2A,B), corresponding to 0, 1, and 2 galactose residues, respectively, while Avastin and Herceptin have only G0 and G1 glycoforms (Figure 2C,D). None had sialylated structures as observed with some mAbs (*6*). To selectively remodel the oligosaccharide of the monoclonal antibodies at Asn 297 in the Fc domain and to obtain a homogeneous population of glycoforms, the IgGs were degalactosylated by β1,4-galactosidase from *S. pneumoniae* for 24 h. After galactosidase treatment, the degree to which the mAbs glycans were degalactosylated was confirmed by MALDI-TOF analysis of the N-glycans after PNGase F treatment. 24 h incubation of 8 mg/mL^{-1} of mAbs with 100 mU units of galactosidase in 50 μL incubation mixture completely converts the IgG in G0 glycoform (Figure 2A′−D′).

Reglycosylation of G0 Glycoform of mAbs Using Either UDP-Gal or UDP-GalNAc as Sugar Donors. The mutant β1,4Gal-T1-Y289L enzyme, in contrast to the wild-type enzyme β1,4Gal-T1, exhibits both β1,4Gal transferase and β1,4GalNAc transferase activities (*24*). Using chemoenzymatic methods, it was shown that the mutant enzyme can transfer the C2-keto-Gal from its UDP-derivative to the GlcNAc residue on the N-glycan chain of an asialo-agalacto IgG molecule (*20*). In the present studies, we investigated the transfer of UDP-sugars to the protein A purified G0 glycoform of Herceptin by the wild-type and a mutant β1,4-galactosyltransferse. Figure 3 shows the MALDI-TOF profile of the oligosaccharide after the transfer of galactose by the wild-type β1,4Gal-T1 (Figure 3A) and the transfer of N-acetylgalactosamine (Figure 3B) by the mutant enzyme β1,4Gal-T1-Y289L to the free GlcNAc residues on the G0 glycoforms of Herceptin (Figure 2D′). Nearly 100% galactosylation of both arms of the N-glycan of the IgG-G0 glycoform

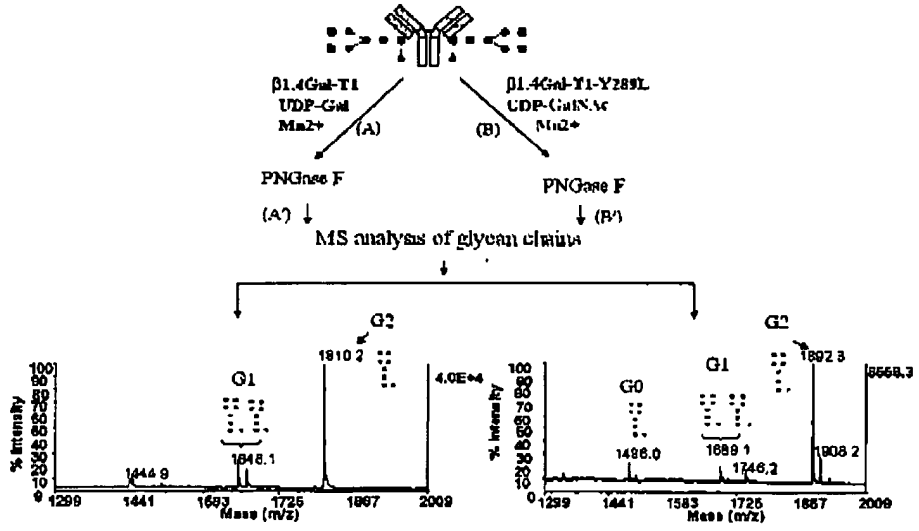

Figure 3. Reglycosylation of β-galactosidase treated mAbs using natural UDP-sugar donors. To the degalactosylated G0 glycoform of the IgG, (A) galactose moiety was transferred from UDP-Galactose using the wild-type enzyme β1,4Gal-T1 or (B) GalNAc moiety was transferred from UDP-GalNAc using the mutant β1,4Gal-T1-Y289L. After overnight incubation, mixtures were treated with PNGase F prior to MS analysis. (A′) After galactose transfer, ions at 1648.1 *m/z* and 1810.2 are assigned to G1 and G2 glycoforms, respectively, and (B′) after GalNAc transfer, ions at 1486.0 *m/z*, 1689.1 *m/z*, and 1892.3 *m/z* are assigned to G0, G1, and G2 glycoforms, respectively.

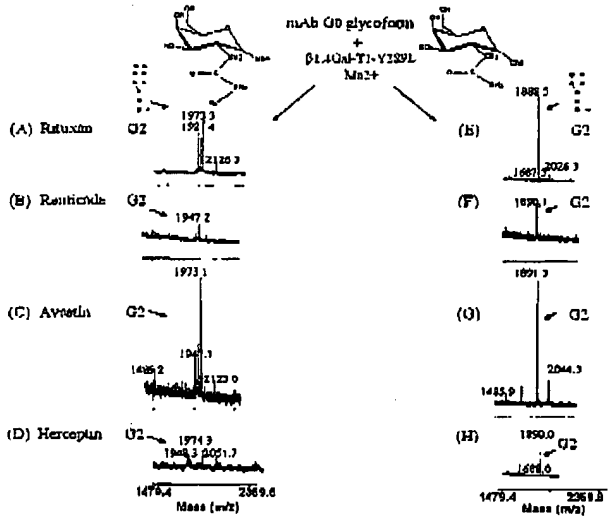

Figure 4. Reglycosylation of G0 glycoform using C-2 modified sugar donors: The mutant enzyme β1,4Gal-T1-Y289L was used to transfer the C-2 modified sugars from their respective UDP-sugar nucleotides to the degalactosylated mAbs; UDP-2-keto-Gal (right panel) and UDP-2-azido-Gal (left panel). MALDI mass analysis after treatment of N-glycans with a mixture of sialydase and β-galactosidase (not shown), gave the same G0 glycoform profile, upon PNGase F treatment, as did the β-galactosidase treatment alone. The transfer of the modified sugar nucleotide was analyzed by mass spectrometry of the modified N-glycans released after PNGase F treatment. β-Galactosidase treated mAbs having a G0 glycoform are fully galactosylated to the G2 glycoform after transfer of GalNaz moiety to the terminal GlcNAc residues showing a peak of 1973 *m/z* (left panel A, C, and D). The G2 glycoform peak at 1947 *m/z* in (B) is due to partial fragmentation of the azido group that can take place during mass spectrometry analysis. The peak at 2126 *m/z* corresponds to the matrix DHB adduct. Right panel (E−H) shows the transfer of C2-keto-Gal moiety to the terminal GlcNAc residues in the G0 glycoform (middle panel). A peak at ~1891 *m/z* corresponds to the G2 glycoform and peaks at 2026 *m/z* and 2044 *m/z* correspond to the DHB adduct. GalNAz (pink stars) and C2-keto-Gal (purple circles).

(Figure 2D′) was observed when 80 pmol of IgG (12 μg) were incubated at 30 °C for 12 h with 120 pmol of β1,4Gal-T1 enzyme (4 μg) at 1.5 mM concentration of the sugar donor substrate UDP-Gal (Figure 3A′). A peak occurred at *m/z* 1810.2, corresponding to the G2 form, and a very small peak at *m/z* 1648.1, corresponding to the G1 form where galactosylation is on either the 1,3 or 1,6 arm of the N-glycan. The transfer of GalNAc (Figure 3B′) by the mutant enzyme β1,4Gal-T1-Y289L to both arms of the G0 glycoform (Figure 2 D′) is observed as a main peak at *m/z* 1892.3 corresponding to the G2 glycoform of the mAb. Minor peaks at *m/z* 1486 and 1689 correspond to the G0 and G1 glycoforms, respectively. Under the conditions described here, the transfer of galactose and of GalNAc to both GlcNAc residues on each arm of the N-linked structure is observed.

Transfer of C2-keto-Gal to the Free GlcNAc Residues on the N-Glycan Chains of mAbs by the Mutant Enzyme β1,4Gal-T1-Y289L. In a previous study, we showed that the transfer of C2-keto-Gal to the N-linked oligosaccharides of the IgG by the mutant β1,4Gal-T1-Y289L (*20*) can be detected by the chemoenzymatic method. In this study, we followed the transfer of C2-keto-Gal to mAbs by the MALDI-TOF of the PNGase F-released oligosaccharides. There is nearly full conversion of 80 pmol of the G0 form of IgG (12 μg) to the G2 form with either C2-GalNAz (Figure 4A−D) or C2-keto-Gal (Figure 4E−G) using 2 mM concentration of sugar donor substrate UDP-C2-GalNAz or UDP-2-keto-Gal, respectively, with ~360 pmol of β1,4Gal-T1-Y289L mutant enzyme (12 μg) in a 25 μL incubation mixture. The appearance of a major peak at *m/z* 1973.5 after the transfer of C2-GalNAz (Figure 4A−D), and at *m/z* 1890.0 after the transfer of C2-keto-Gal (Figure 4E−G), shows that the transfer occurs under these conditions on both arms of the G0 glycoform.

Biotinylation of the Glycosylated IgG. The transfer of C2-keto-Gal has been followed by coupling the biotinylated aminooxy biotin to the ketone group at the C2 position of the galactose (Figure 5A) and detected by a sensitive chemiluminescence assay (*19*−*21*). MALDI-TOF analysis of the oligosaccharides released after PNGase F treatment of the C2-keto-galactosylated mAbs showed a major peak at *m/z* 2519, corresponding to the G2 glycoform of the antibodies (Figure 5B) having 4 molecules of C2-keto-Gal transferred per IgG molecule. The conjugated proteins were also analyzed by SDS-PAGE, followed by Western blotting and detection by the streptavidin-HRP technique, as previously described (*19*−*21*). The lack of chemiluminescence band in the Western blot analysis after PNGase F treatment of the mAbs (Figure 5C) supports the conclusion that the biotinylated aminooxy ligand is linked only to the C2-keto-Gal modified N-glycan chain of the IgG heavy chain.

Comparing the Binding of Native and Modified Avastin to VEGF by the ELISA Method. We used an indirect ELISA method described previously (*26*) to analyze the binding of native and modified Avastin to VEFG immobilized on a microtiter plate surface. Remodeling of the Fc N-glycans of Avastin did not change the binding to VEGF, when compared to the binding of the native mAb (Figure 6).

Herceptin Labeling with Alexa Fluor 488 C_5−Aminooxyacetamide. Since C2-keto galactosylated mAbs could be successfully coupled to aminooxy containing ligands (Figure 5B,C) without affecting the antigen binding, as observed by the ELISA method (Figure 6), we linked the C2-keto-Gal modified Herceptin (Figure 4H) to the Alexa Fluor 488 C_5-aminooxyacetamide and analyzed the protein by fluorescence imaging of the SDS-PAGE (Figure 7A), and subsequent staining of the gel with Coomassie blue (Figure 7B). Results from the fluorescence imaging indicate that there is a selective labeling of the heavy chain of the mAb, and this signal is almost lost following PNGase F treatment of the fluorescent-tagged antibody, which removes the N-linked sugars in the Fc domain of the IgG (Figure 7A). No fluorescence was detected at other protein bands seen after Coomassie blue staining of the SDS-PAGE gel (Figure 7B). Conjugation of Alexa Fluor 488 to enzyme-modified Herceptin was further confirmed using a spectrofluorometer (model FluoroMax 3, HORIBA Jobin Yvon Inc., NJ) to measure fluorescence at Ex/Em 494/518 nm (not shown).

Effect of Herceptin Modification(s) on its Binding to HER2 Receptor. To evaluate the effect of carbohydrate modifications in Herceptin on its biological activity, we examined its binding to HER2 receptor using a human breast adenocarcinoma cancer cell line (SKBR-3) that overexpresses this receptor. MDA-MB-468 cells, that do not express the HER2 receptor, were used as controls. The immunostaining with Alexa Fluor 488 C_5-aminooxyacetamide-conjugated Herceptin or modified Herceptin was performed as described in the Methods section. The results are presented in Figures 8 and 9, respectively.

Alexa-conjugated Herceptin showed binding only to SKBR-3 cells (receptor expressing) (Figure 8B). In contrast, under identical conditions, we did not observe any binding above background for MDA-MB-468 cells (receptor negative) (Figure 8A). Therefore, the conjugation of Alexa Fluor 488 and

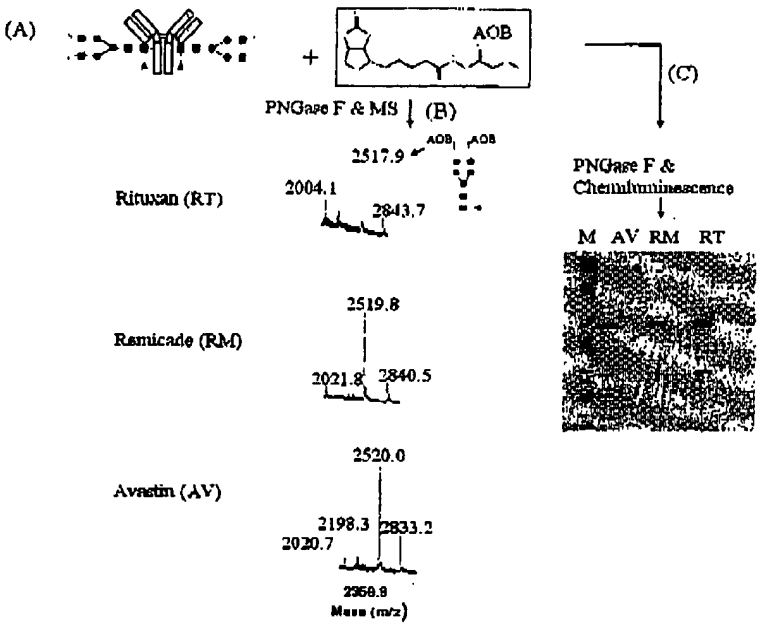

Figure 5. Detection by chemiluminescence technique of the transfer of the C2-keto-Gal to free GlcNAc residues on the N-glycan chains of mAbs. (A) The C2-keto-Gal has been transferred to the free GlcNAc residues in the N-linked carbohydrate of IgG. (B) Detection of the transferred C2-keto-Gal is accomplished by linking to aminooxy biotin followed by PNGase F treatment and MS analysis of the N-glycans. The peaks at 2517.9 *m/z*, 2519.8 *m/z*, and 2520.0 *m/z* correspond to AOB being transferred to both arms of N-glycans carrying C2-keto-Gal in Rituxan (RT), Remicade (RM), and Avastin (AV), respectively. The peaks at 2843.7 *m/z*, 2840.5 *m/z*, and 2833.2 *m/z* correspond to AOB transferred to the sugars at the reducing end. (C) The linked AOB-C2-keto-Gal of AV, RM, and RT is also detected on Western blots by chemiluminescence. In contrast to the samples that were not treated with PNGase F (−), the samples treated with PNGase F (+) showed no chemiluminescence. C2-keto-Gal (purple circles).

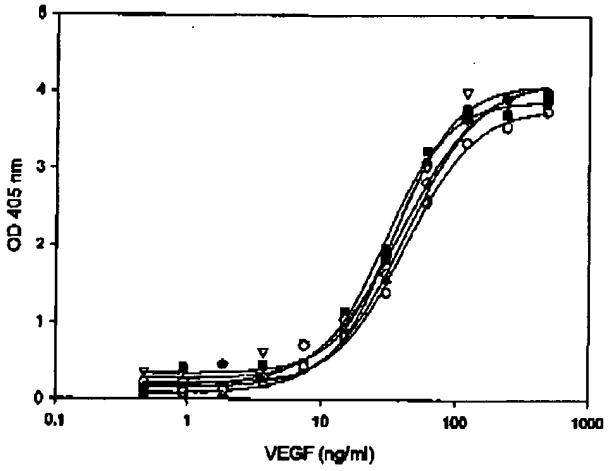

Figure 6. VEGF ELISA comparing the binding of modified Avastin. Avastin, native or modified, was bound to VEGF immobilized on a microtiter plate surface, and was detected with an antihuman Fc antibody conjugated to alkaline phosphatase. The transfer of various substrates by the mutant β1,4Gal-T1-Y289L, namely, GalNAc and C2-keto-Gal to Avastin did not perturb the binding to VEGF. Aminooxy biotin is also linked to the modified C2-keto-Gal without perturbing the Fab domain. (●) Avastin native. (▽) Degalactosylated Avastin. (□) Degalactosylated Avastin+ GalNAc. (◇) Degalactosylated Avastin+ no UDP-GalNAc+β1,4Gal-T1-Y289L. (▣) Degalactosylated Avastin+ C2-keto-Gal. (○) Degalactosylated Avastin+ C2-keto-Gal +AOB.

Herceptin did not have any effect on its receptor-binding activity, suggesting that these conjugates may serve as suitable tools for imaging.

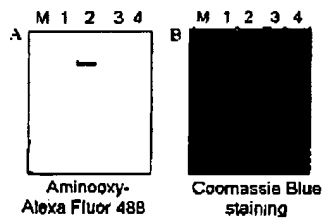

Figure 7. Herceptin labeling with Alexa Fluor 488 C_5-aminooxy acetamide. C2-keto-Gal modified Herceptin was conjugated with Alexa Fluor 488 C_5-aminooxyacetamide and analyzed by SDS-PAGE (see Materials and Methods). (A) Fluorescence imaging of the SDS-PAGE. (B) The gel was subsequently stained with Coomassie blue. (M) Kaleidoscope standard, (1) β1,4Gal-T1-Y289L, (2) C2-keto-Gal modified Herceptin conjugated to Alexa Fluor 488 C_5-aminooxy acetamide, (3) C2-keto-Gal modified Herceptin conjugated to Alexa Fluor 488 C_5-aminooxyacetamide digested with PNGase F, and (4) PNGase F.

Next, we examined the effect of carbohydrate modifications in Herceptin, other than Alexa Fluor 488, on the binding to the HER2 receptor expressing cells. We performed cell sorting experiments using indirect immunostaining (using the FITC-conjugated goat antihuman IgG, see Methods). The results are presented in Figure 9. SKBR-3 (receptor positive) cells bound to native and to modified Herceptin conjugates with similar efficiency (Figure 9 F−H). In contrast, FITC-conjugated goat antihuman IgG alone did not show any significant binding above background (Figure 9 E). Our data also show that MDA-MB-468 cells (receptor negative) did not bind to Herceptin (or modified Herceptin) above background (Figure 9A−D). Therefore, carbohydrate modifications and/or subsequent conjugation

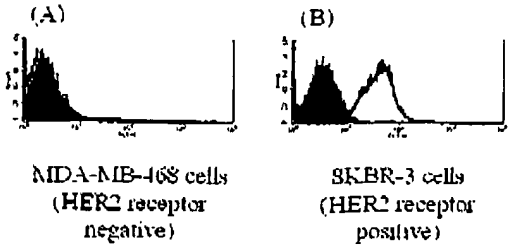

Figure 8. Binding of Alexa Fluor 488 conjugated Herceptin to HER2-receptor expressing cells. Herceptin was enzymatically modified with C2-keto-Gal and conjugated with Alexa Fluor 488 C_5-aminooxyacetamide. Alexa-conjugated Herceptin (4 μg) was incubated with 10^6 cells/100 μL PBS−FBS for 60 min at 4 °C while shaking in the dark. The cells were washed twice with PBS−BSA and resuspended in 1 mL PBS−BSA. Samples were analyzed by FACS (see Materials and Methods). (A) MDA-MB-468 control cells and (B) SKBR-3 cells. The purple area indicates the background value. The empty area defines the fluorescence of Herceptin conjugated Alexa Fluor 488 C_5-aminooxyacetamide (green line).

via the introduced functional groups do not alter their receptor binding activity.

DISCUSSION

The mAbs are used as powerful therapeutic agents for the treatment of various diseases. They can be used "naked" with their inherent effector functions or as targeting vehicles. Here, we used an enzymatic method (*19*−*21*) for the production of the conjugated targeting vehicle of mAbs that has the advantage (I) of being site-specific and accessible for conjugation; (II) the cargo does not interfere with or modify the Fab domain; (III) the conjugation of a cargo molecule to a mAb makes it possible to direct the cargo to the desired site; (IV) mAb conjugated with fluoroprobes via glycan chains are suitable probes for imaging and clinical applications.

We have previously shown that the mutant enzyme β1,4Gal-T1-Y289L can transfer GalNAc and galactose from its UDP-derivatives to GlcNAc residues present at the nonreducing end of proteins (*24*). We have also shown that this mutant can transfer modified sugars such as 2-keto-Gal from their UDP derivatives for detection of O-GlcNAc modifications in proteins (*19*) or detection of free GlcNAc residues at the nonreducing end of the N-glycans of proteins (*20*). Our findings led us to propose that two glycoproteins having modified sugars with unique chemical handles could be conjugated via linkers having orthogonal chemical reactive groups, allowing the design of novel mAb-drug conjugates and imaging agents (*27*).

The present study extends our previous work by showing: (1) Fc N-glycans of native therapeutic antibodies are highly heterogeneous; (2) it is possible to modify the therapeutic monoclonal antibodies from a heterogeneous to a homogeneous glycoform population; (3) the modification at the N-linked oligosaccharide occurs in a site-specific manner without compromising the antigen binding site; and (4) we have been able to produce mAb-fluoroprobes conjugates for imaging applications.

The glycosylation profile of recombinant mAbs has been well-characterized and is known to be highly heterogeneous with respect to core fucosylation, terminal sialylation, and galactosylation (*28*). The difference between the observed glycoform types depends on the enzymes/host cell type, substrates, and culture conditions utilized for their production. The MALDI-TOF analysis of PNGase F-released oligosaccharides of the mAbs used in our study concur with the published data (*28*). These N-glycans are core-fucosylated with differences in the terminal sugars having a mixture of G0, G1, and G2 glycoforms. None of the mAb used here had terminal sialic acids. Previously, we showed that the C2-keto-Gal from its UDP derivative was transferred by β1,4-Gal-T1-Y289L to free GlcNAc residues on the N-linked glycan chains of a heterogeneous population of IgG molecules (*20*). An important issue in this study was the transfer of the modified sugar to each available GlcNAc present in a homogeneous mAb glycoform population. In order to obtain a population of mAb having a single G0 glycoform with 2 mol of GlcNAc available for transfer to each IgG heavy chain, we tested galactosidases from various sources to remove the

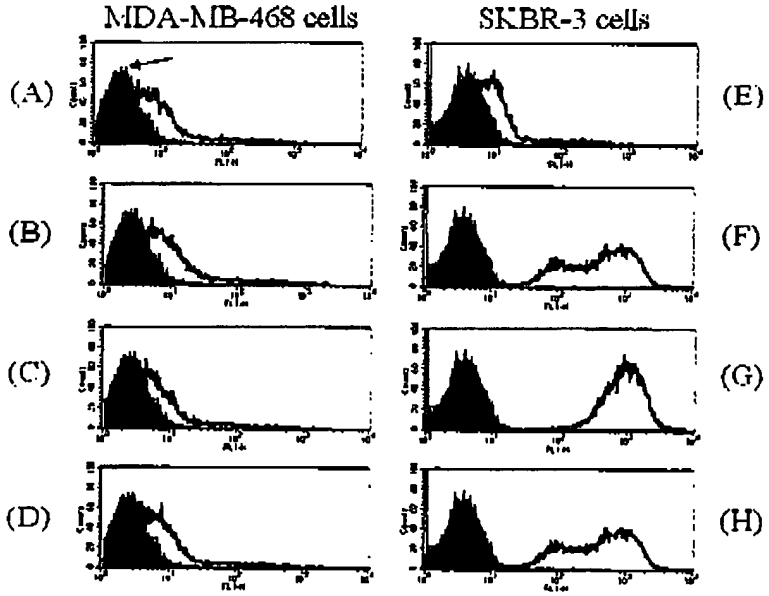

Figure 9. Effect of carbohydrate modification of Herceptin on its binding to HER2-receptor expressing cells. Herceptin was subjected to various enzyme treatments (see Materials and Methods). Native and modified Herceptin molecules were incubated with MDA-MB-468 cells or SKBR-3, followed by incubation with FITC-conjugated goat antihuman IgG. The samples were analyzed by FACS (see Methods section). (A,E) secondary antibody alone; (B,F) Herceptin untreated; (C,G) Herceptin treated with β-galactosidase; (D,H) Herceptin treated with β-galactosidase + C2-keto-Gal. The arrow indicates background fluorescence of cells in the absence of antibodies.

nonreducing end galactose residues in the G1 and G2 glycoforms. Only after using β1,4 galactosidase from *Streptococcus pneumoniae* (*9*), we were able to show, by MALDI-TOF analysis of the PNGase F-released carbohydrates, a single-type G0 glycoform. The β1,4 galactosidase from other sources did not yield 100% fully degalactosylated glycoforms. Having demonstrated the production of a single glycoform, the G0 form, by enzymatic degalactosylation, we explored the transfer of the modified sugar and the coupling of the ketone handle to aminooxy-biotin, which was then detected by MALDI-TOF analysis of N-glycan chains or by chemiluminescence (*20*), or coupling to aminooxi Alexa Fluor 488, which was detected by a fluorescence technique after SDS-PAGE and FACS analysis. Both chemiluminescence and fluorescence analysis showed that the method of coupling described here is highly specific; almost all the chemiluminescence and fluorescence from the antibody conjugates were associated with binding to the heavy chain. Thus, the cargo is linked in a site-directed manner and only occurs where the sugar binds in the Fc domain of the IgG.

This study also shows that the linking of the target agent, either biotin or Alexa Fluor 488, to the mAb via the N-linked carbohydrates does not modify the antibody affinity for the antigen. One important goal in using this conjugation method is to generate a delivering agent: an antibody−cargo conjugate that will not interfere in its binding to the target cells. The results from ELISA assays using native mAb showed no detectable loss of antigen (Ag) binding activity. Moreover, all antibodies described in this study, obtained either by removal or by addition of sugars, when conjugated via glycan chains, did not show a loss of Ag binding activity. The Ag binding activity of the Fc-N-glycan modified antibodies was comparable to the results obtained with the native mAb. Also, the results obtained by FACS are consistent with the ELISA assay in that the binding of mAb conjugate to HER-2-expressing cells was not affected by the modification.

In conclusion, the present study describes an enzymatic method for conjugation of monoclonal antibodies at a unique site, away from the Ag binding domain. This method using modified sugars for conjugation provides a specific labeling with 4 molecules of fluorescent dyes if the IgG molecule has free GlcNAc residues available on the Fc N-glycan chains. To our knowledge, this is the first report on the site-specific conjugation of fluoroprobes to the N-linked glycans of intact mAb using an enzymatic method. Other laboratories have reports on the use of molecular imaging probes using mAbs; however, they use chemical conjugation methods, which lead to random labeling (*29*, *30*). Thus, this conjugated fluoroprobe may be used as a probe for imaging and clinical applications.

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LITERATURE CITED

(1) Brumlik, M. J., Daniel, B. J., Waehler, R., Curiel, D. T., Giles, F. J., and Curiel, T. J. (2008) Trends in immunoconjugate and ligand receptor-based targeting development for cancer therapy. *Expert Opin. Drug Deliv. 5*, 87–103.

(2) Ross, J. S., Schenkein, D. P., Pietrusko, R., Rolfe, M., Linette, G. P., Stec, J., Stagliano, N. E., Ginsburg, G. S., Symmans, W. F., Pusztai, L., and Hortobagyi, G. N. (2004) Targeted therapies for cancer. *Am. J. Clin. Pathol. 122*, 598–609.

(3) Hamann, P. R. (2005) Monoclonal antibody-drug conjugates. *Expert Opin. Thera. Patents 15*, 1087–1103.

(4) Carter, P. J., and Senter, P. D. (2008) Antibody-drug conjugates for cancer therapy. *Cancer J. 14*, 154–169.

(5) Trail, P. A., King, H. D., and Dubowchik, G. M. (2003) Monoclonal antibody drug immunoconjugates for targeted treatment of cancer. *Cancer Immunol. Immunother. 52*, 328–337.

(6) Arnold, J. N., Dwek, R. A., Rudd, P. M., and Sim, R. B. (2006) Mannan binding lectin and its interaction with immunoglobulins in health and in disease. *Immunol. Lett. 106*, 103–110.

(7) Jefferis, R. (2005) Glycosylation of recombinant antibody therapeutics. *Biotechnol. Prog. 21*, 11–16.

(8) Holland, M., Yagi, H., Takahashi, N., Kato, K., Savage, C. O., Goodall, D. M., and Jefferis, R. (2006) Differential glycosylation of polyclonal IgG, IgG-Fc and IgG-Fab isolated from the sera of patients with ANCA-associated systemic vasculitis. *Biochim. Biophys. Acta 1760*, 669–677.

(9) Hodoniczky, J., Zheng, Y. Z., and James, D. C. (2005) Control of recombinant monoclonal antibody effector functions by Fc N-glycan remodeling in vitro. *Biotechnol. Prog. 6*, 1644–1652.

(10) Shinkawa, T., Nakamura, K., Yamane, N., Shoji-Hosaka, E., Kanda, Y., Sakurada, M., Uchida, K., Anazawa, H., Satoh, M., Yamasaki, M., Hanai, N., and Shitara, K. (2003) The absence of fucose but not the presence of galactose or bisecting *N*-acetylglucosamine of human IgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. *J. Biol. Chem. 278*, 3466–3473.

(11) Sharkey, R. M., and Goldenberg, D. M. (2008) Use of antibodies and immunoconjugates for the therapy of more accessible cancers. *Adv. Drug Delivery Rev. 60*, 1407–1420.

(12) Byrne, J. D., Betancourt, T., and Brannon-Peppas, L. (2008) Active targeting schemes for nanoparticle systems in cancer therapeutics. *Adv. Drug Delivery Rev. 60*, 1615–1626.

(13) Garnett, M. C. (2001) Targeted drug conjugates: principles and progress. *Adv. Drug Delivery Rev. 53*, 171–216.

(14) O'Shannessy, D. J., and Quarles, R. H. (1971) Labeling of the oligosaccharide moieties of immunoglobulins. *J. Immunol. Methods 99*, 153–161.

(15) Van Lenten, L., and Ashwell, G. (1971) Studies on the chemical and enzymatic modifications of glycoproteins. A general method for the tritiation of sialic-acid containing glycoprotein. *J. Biol. Chem. 246*, 1889–1894.

(16) Chua, M., Fan, S., and Karush, F. (1984) Attachment of immunoglobulin to liposomal membrane via protein carbohydrate. *Biochim. Biophys. Acta 800*, 291–300.

(17) Morell, A. G., Van den Hamer, C. J., Scheinberg, I. H., and Ashwell, G. (1966) Physical and chemical studies on ceruloplasmin. IV. Preparation of radioactive, sialic acid-free ceruloplasmin labeled with tritium on terminal D-galactose residues. *J. Biol. Chem. 241*, 3745–3749.

(18) Wilchek, M., and Bayer, E. A. (1984) The avidin-biotin complex in immunology. *Immunol. Today 5*, 39–43.

(19) Khidekel, N., Arndt, S., Lamarre-Vincent, N., Lippert, A., Poulin-Kerstien, K. G., Ramakrishnan, B., Qasba, P. K., and Hsieh-Wilson, L. C. (2003) A chemoenzymatic approach toward the rapid and sensitive detection of *O*-GlcNAc posttranslational modifications. *J. Am. Chem. Soc. 125*, 16162–16163.

(20) Boeggeman, E., Ramakrishnan, B., Kilgore, C., Khidekel, N., Hsieh-Wilson, L. C., Simpson, J. T., and Qasba, P. K. (2007)

Direct identification of nonreducing GlcNAc residues on N-glycans of glycoproteins using a novel chemoenzymatic method. *Bioconjugate Chem. 18*, 806–814.

(21) Ramakrishnan, B., Boeggeman, E., and Qasba, P. K. (2007) Novel method for in vitro O-glycosylation of proteins: application for bioconjugation. *Bioconjugate Chem. 18*, 1912–1918.

(22) Qasba, P. K., Boeggeman, E., and Ramakrishnan, B. (2008) Site-specific linking of biomolecules via glycan residues using glycosyltransferases. *Biotechnol. Prog. 24*, 520–526.

(23) Boeggeman, E., Ramakrishnan, B., and Qasba, P. K. (2003) The *N*-terminal stem region of bovine and human β1,4-galactosyltransferase I increases the in vitro folding efficiency of their catalytic domain from inclusion bodies. *Prot. Expres. Purif. 30*, 219–220.

(24) Ramakrishnan, B., and Qasba, P. K. (2002) Structure-based design of beta-1,4-galactosyltransferase-I (beta 4Gal-T1) with equally efficient N-Acetylgalactosaminyltransferase activity: point mutation broadens beta 4Gal-T1 donor specificity. *J. Biol. Chem. 277*, 20833–20839.

(25) Snovida, S. I., and Perreault, H. (2007) A 2,5-dihydroxybenzoic acid/N, N-dimethylaniline matrix for the analysis of oligosaccharides by matrix-assisted laser desorption/ionization mass spectrometry. *Rapid Commun. Mass Spectrom. 21* (24), 3711–3715.

(26) Bock, F., Onderka, J., Dietrich, T., Bachmann, B., Kruse, F. E., Paschke, M., Zahn, G., and Cursiefen, C. (2007) Bevacizumab as a potent inhibitor of inflammatory corneal angiogenesis and lymphangiogenesis. *Invest. Ophthalmol. Vis. Sci. 48*, 2545–2552.

(27) Ramakrishnan, B., Boeggeman, E., and Qasba, P. K. (2008) Applications of glycosyltransferases in the site-specific conjugation of biomolecules and the development of a targeted drug delivery system and contrast agents for MRI. *Expert Opin. Drug Delivery 5*, 149–153.

(28) Raju, T. S., Briggs, J. B., Borge, S. M., and Jones, A. J. (2000) Species-specific variation in glycosylation of IgG: evidence for the species-specific sialylation and branch-specific galactosylation and importance for engineering recombinant glycoprotein therapeutics. *Glycobiology 10*, 477–486.

(29) Hama, Y., Urano, Y., Koyama, Y., Choyke, P. L., and Kobayashi, H. (2007) Activatable fluorescent molecular imaging of peritoneal metastases following pretargeting with a biotinylated monoclonal antibody. *Cancer Res. 67*, 3809–3817.

(30) Koyama, Y., Barrett, T., Hama, Y., Ravizzini, G., Choyke, P. L., and Kobayashi, H. (2007) In vivo molecular imaging to diagnose and subtype tumors through receptor-targeted optically labeled monoclonal antibodies. *Neoplasia 9*, 1021–1029.

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Multiple Site-Specific *in Vitro* Labeling of Single-Chain Antibody

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For multiple site-specific conjugations of bioactive molecules to a single-chain antibody (scFv) molecule, we have constructed a human anti HER2 receptor, scFv, with a C-terminal fusion polypeptide containing 1, 3, or 17 threonine (Thr) residues. The C-terminal extended fusion polypeptides of these recombinant scFv fusion proteins are used as the acceptor substrate for human polypeptide-α-N-acetylgalactosaminyltransferase II (h-ppGalNAc-T2) that transfers either GalNAc or 2-keto-Gal, a modified galactose with a chemical handle, from their respective UDP-sugars to the side-chain hydroxyl group of the Thr residue(s). The recombinant scFv fusion proteins are expressed in *E. coli* as inclusion bodies and *in vitro* refolded and glycosylated with h-ppGalNAc-T2. Upon protease cleavage, the MALDI-TOF spectra of the glycosylated C-terminal fusion polypeptides showed that the glycosylated scFv fusion protein with a single Thr residue is fully glycosylated with a single 2-keto-Gal, whereas the glycosylated scFv fusion protein with 3 and 17 Thr residues is found as an equal mixture of 2−3 and 5−8 2-keto-Gal glycosylated fusion proteins, respectively. These fusion scFv proteins with the modified galactose are then conjugated with a fluorescence probe, Alexa488, that carries an orthogonal reactive group. The fluorescence labeled scFv proteins bind specifically to a human breast cancer cell line (SK-BR-3) that overexpresses the HER2 receptor, indicating that the *in vitro* folded scFv fusion proteins are biologically active and the presence of conjugated multiple Alexa488 probes in their C-terminal end does not interfere with their binding to the antigen.

INTRODUCTION

Conjugation of monoclonal antibodies with bioactive molecules such as toxins, drugs, and radioisotopes is an emerging strategy in the treatment and diagnosis of cancer (*1*−*5*). Not only is the conjugation of a multiple number of bioactive molecules to a single antibody molecule desired, but also, site-specific conjugation of these molecules is important. The former enhances the sensitivity of detection and better treatment of cancer, while the latter enables the production of a homogeneous antibody−drug complex that ensures the full activity of both the antibody and the bioactive molecule in an antibody−drug conjugate. Multiple site-specific conjugation has been achieved by engineering the target protein, either by the introduction of free Cys residues (*6*, *7*) or by introducing genetically encoded aldehydes at either terminal end of the protein or by site-directed introduction of azido/alkynyl-tagged methionine analogues into proteins (*8*−*10*). However, protein with free Cys residue(s) needs special handling to prevent its free Cys residues from undergoing undesired oxidation while in the process of introducing the methionine analogue all the methionine residues in the protein are modified. Not many site-specific conjugation methods offer multiple site conjugations.

We have developed a unique, site-specific conjugation method using the mutant galactosyltransferase enzymes, where we first enzymatically transfer 2-acetonyl-2-deoxy-galactose (2-keto-Gal) sugar with a unique chemical handle to a specific sugar moiety, βGlcNAc or βGal1-4GlcNAc, present at the nonreducing end of the glycan of the glycoprotein; then, the chemical handle present in the modified sugar is used for site-specific conjugation with biologically important molecules having a corresponding orthogonal chemical group (*11*−*14*). Using this method, we have recently shown that the biantenary *N*-glycans of a therapeutic IgG molecule have been used as the substrate for the mutant Y298L-Gal-T1 enzyme to transfer 2-keto-Gal sugar and further conjugated with bioactive molecules such as biotin to both arms of the biantenary *N*-glycans, thus producing the native IgG molecule with four biotin molecules site-specifically conjugated (*12*, *14*). However, this method requires the presence of a specific sugar moiety of a glycan chain in the glycoprotein and cannot be used for nonglycosylated proteins, such as single-chain antibodies or bacterial toxins expressed in *E. coli*.

For proteins that lack glycosylation motifs, we have recently developed a method using the human polypeptide-α-*N*-acetylgalactosaminyltransferase II enzyme (h-ppGalNAc-T2), which transfers *N*-acetylgalactosamine sugar (GalNAc) from UDP-GalNAc to Thr/Ser residues on an acceptor polypeptide that is at least 11 amino acids long (*15*, *16*). In this method, we have engineered the acceptor polypeptide substrate of the h-ppGalNAc-T2 with one Thr residue as a fusion peptide at the C-terminus of a bacterial glutathione-*S*-transferase (GST), which is not normally glycosylated. We used this fusion polypeptide moiety as an acceptor substrate for the h-ppGalNAc-T2 to transfer modified Gal with chemical handle such as 2-keto-Gal or GalNAz, thus enabling us to site-specifically conjugate bioactive molecules with a corresponding orthogonal chemical group (*15*). Using this method, in the present study we demonstrate that an anti human HER2 receptor single-chain antibody (anti HER2 scFv) with a fusion peptide containing more than one Thr residue at its C-terminal end can be successfully glycosylated with 2-keto-Gal and then conjugated

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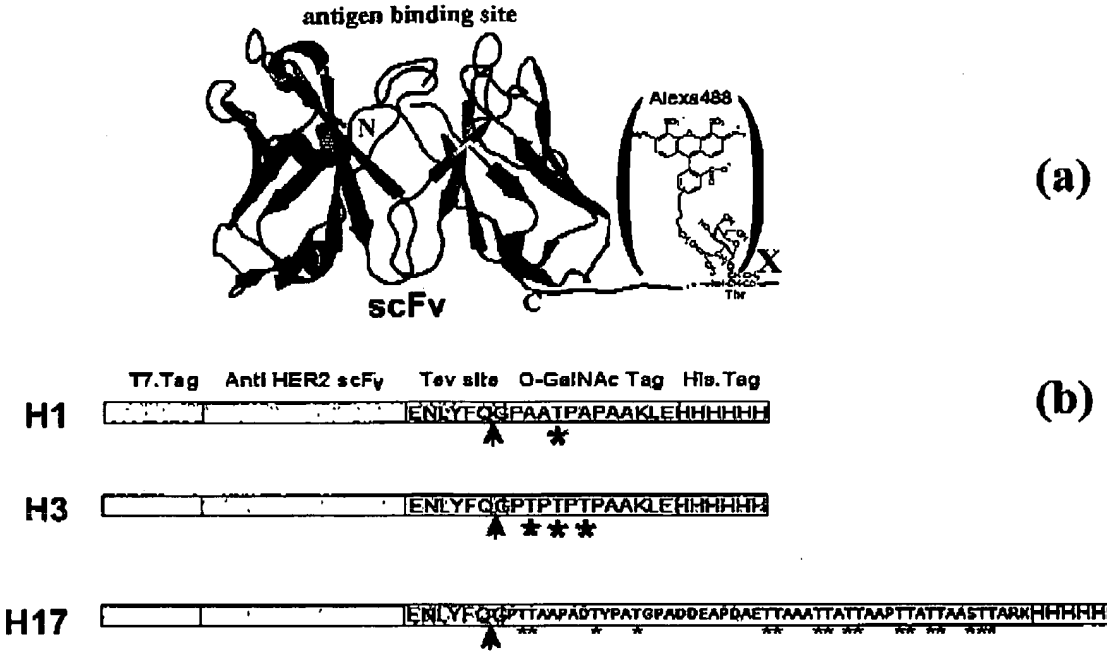

Figure 1. (a) Schematic molecular structure of the Alexa488-conjugated scFv fusion protein. In the present study a single-chain antibody against human HER2 receptor (anti HER2 scFv) with *x* number of Alexa488 molecules conjugated through the modified sugar is named Hx. The anti HER2 scFv protein has been designed to have specific 1 to 17 O-glycosylation sites. (b) Schematic diagram of the design of anti HER2 scFv proteins with multiple glycosylation sites. The antihuman HER2 receptor single-chain antibody (anti HER2 scFv) expressed in *E. coli* contains a N-terminal T7-tag, a C-terminal Tev protease-cleavable O-glycosylation tag, and an His tag. The proteins with 1, 3, and 17 Thr residues in their O-glycosylation tags (shown as stars at their bottom) are named H1, H3, and H17, respectively. The Tev protease cleavage site is indicated by an arrow. In the presence of Mn^{2+}, the polypeptide-α-*N*-acetylgalactosaminyltransferase II (h-ppGalNAc-T2) enzyme transfers GalNAc or 2-keto-Gal sugar from their UDP derivatives to the side-chain hydroxyl group of the Thr residue present in the O-glycosylation tag.

with Alexa488, a fluorescence probe. Furthermore, our cell surface immunostaining analysis of HER2-receptor expressing SK-BR-3 cells (by fluorescent-activated cell sorting [FACS]) shows that antibody binding to the HER2 receptor is not affected by the one or more Alexa488 molecules present at the C-terminal end of these scFv molecules.

EXPERIMENTAL PROCEDURES

Anti HER2 Single-Chain Antibody Gene Construction and Expression in *E. coli*. The antihuman HER2 scFv gene was constructed from a fully human Fab isolated from a large native phage display Fab library, using HER2 ectodomain as a target (Zhu, Z., and Dmitrov, D., unpublished data). For the construction of H1 and H3 scFv proteins, the anti HER2 scFv gene was repeatedly amplified with the 5-primer, 3-primer-1, and 3-primer2, which introduces a 17-amino-acid peptide as a C-terminal extension following a Tev protease cleavage site. A final PCR amplification with either 3-H(1) or 3-H(3) primer results in a product with a C-terminal O-glycosylation tag without a stop codon, having *Eco*RI and Xho I restriction enzyme sites at their 5′ and 3′ termini, respectively. This construction uses the stop codon at the 3′ terminal in the vector after the His-tag.

5-primer: GCC CCG *GAA TTC* GGG CGC GGC GAA GTG CAG CTG GTG CAG TCT GGA GCA GAG GTG AAA AAG.

3-Primer 1: GCC TTG GAA GTA AAG GTT TTC TAG GAC GGT GAC CTT GGT CCC AGA TCC GAA GAC AGC AAT.

3-Primer 2: TTT AGC TGC CGG TGC GGG AGT AGC TGC AGC GCC TTG GAA GTA AAG GTT TTC.

3-H1 Primer: GTG GTG GTG *CTC GAG* TTT AGC TGC CGG TGC GGG ACT AGC TGC AGG GCC.

3-H3 Primer: GTG GTG GTG *CTC GAG* TTT AGC TGC CGG TGT GGG AGT AGG TGT AGG GCC.

The PCR-amplified product was digested with *Eco*RI and Xho I restriction enzymes and ligated with the pET23a vector digested with the same enzymes. The ligated vector was transfected into XL2 supercompetent cells. The ampicillin-resistant clones were screened for the presence of the anti HER2 scFv gene in their plasmid DNA between their *Eco*RI and Xho I restriction sites. The N-terminal T7-tag, which enhances the protein expression in *E. coli* and the C-terminal His × 6 tag, followed by a stop codon, comes from the pET23a vector DNA. For the construction and expression of H17 scFv protein, the cDNA for the scFv protein was first cloned into the pET23a vector between the *Eco*RI and Hind III restricted enzyme sites without the stop codon. This cloning was followed by inserting between the Hind III and Xho I site a cDNA sequence coding for a Tev protease cleavage site and the 50 amino-acid-long polypeptide of the O-glycosylation region of the human small breast epithial mucin protein (BC111421) (*17*) without the stop codon. This construction uses the stop codon at the 3′ terminal in the vector after the His-tag, similar to H1 and H3 scFv gene constructs. The positive clones were sequenced and transfected into Rosetta (DE3) LysS competence cells for expression of the protein.

***In Vitro* Folding of Inclusion Bodies of Anti-HER2 scFv Protein from *E. coli*.** The Rosetta (DE3) LysS cells containing the anti HER2 scFv cDNA sequence in the pET23a vector were grown to an optical density of 0.7−0.8 and then induced with IPTG. The inclusion bodies were purified from the bacterial pellet as described earlier (*18*). From 1 L of bacterial culture, 60 to 70 mg of protein were obtained as inclusion bodies. The *in vitro* folding of anti HER2 scFv was

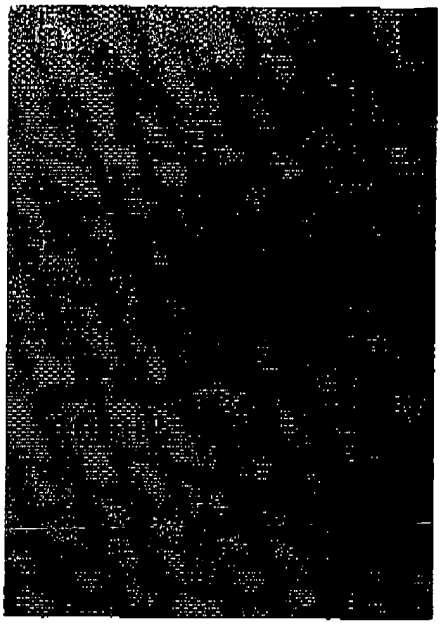

Figure 2. SDS-PAGE gel analysis of the unglysoylated (H1- and H17-) and 2-keto-Gal glycosylated (H1+, H3+, and H17+) proteins: The unglycosylated H1 and H17 proteins are labeled as H1- and H17-, respectively, while the 2-keto-Gal glycosylated H1, H3, and H17 are labeled as H1+, H3+, and H17+, respectively. The h-ppGalNAc-T2 in the glycosylation mixture is also labeled. MALDI-TOF analysis of the H1+, H3+, and H17+ anti HER2 scFv proteins show that these proteins carry 2-keto-Gal (see below). The large difference in the mobility between unglycosylated and glycosylated H17 protein is due to glycosylation, however the microhetrogenity in its glycosylation is not observed (see below). Similarly the mobility difference between the H1 unglycosylated protein and the H1+ and H3+ glycosylated protein may be due to their glycosylation.

carried out in a way similar to that of β4Gal-T1 (*18*). Typically, 100 mg of sulfonated protein was folded for 48 h in 1 L of folding solution that contains oxido-shuffling agents and 500 mM arginine HCl. After refolding the protein, the folding solution was extensively dialyzed against water. During dialysis, the misfolded protein precipitated out, while the folded protein remained soluble. The soluble protein was concentrated to a concentration of 1 mg/mL on an Amicon stirred cell, using a YM-10 membrane, and further purified on a Ni-column. There was no significant loss of protein observed during concentration or during Ni-column purification steps, as estimated by measuring the protein concentration using the Bradford method (BioRad). Nearly 20 mg of folded anti HER2 scFv protein was obtained from 1 L of folding solution. The C-terminal glycosylation peptide tag was released from a 5 μg protein by Tev protease cleavage and analyzed by mass spectrometry.

Glycosylation and Alexa488 Conjugation of Anti HER2 scFv. The cloning, expression, and refolding of the h-ppGalNAc-T2 enzyme has been previously published (*15*). UDP-GalNAc was purchased from Sigma chemicals while the UDP-2-keto-Gal was synthesized in-house. Forty micrograms of anti HER2 scFv fusion protein—H1, H3, or H17—were incubated overnight at room temperature 20 °C with 20 μg of h-ppGalNAc-T2 in the presence of 25 mM Tris-HCl (pH 8.0), 10 mM $MnCl_2$, and 0.5 mM UDP-sugar (UDP-GalNAc, or UDP-2-keto-Gal,) in a total volume of 100 μL (Figure 2). The amount of h-ppGalNAc-T2 enzyme used in these experiments to fully glycosylate the fusion peptide using UDP-GalNAc as the donor substrate was first determined in separate experiments. To remove the entire reaction buffer, the glycosylated anti HER2 scFv protein was precipitated with 40% ammonium sulfate and desalted three times by repeated dilution to 500 μL with water and concentrated each time to 40 μL using microcon centrifuge filters with 10K molecular weight cutoff. The salt-free glycosylated anti HER2 scFv protein was reconstituted in 40 μL water, and an aliquot (2 μL) was cut with Tev protease and analyzed by mass spectrometry (MALDI-TOF).

The entire 40 μg of the desalted 2-keto-Gal glycosylated anti HER2 ScFv protein was conjugated in 60 μL with 75 mM sodium acetate buffer (pH 5.0) and 8 μL *N*-aminoxymethylcarbonylhydrazino-Alexa488 (1 mg/mL in DMSO). Nearly 1000-fold excess of Alexa-488 molecules, more than the manufacturer (Invitrogen) suggested amount, was used in this conjugation reaction. The reaction was carried out in the dark, overnight, at room temperature. The conjugated protein was precipitated with 40% ammonium sulfate, redissolved in water to a 1 μg/μL concentration and used for cell binding. The presence of Alexa488 was confirmed by visualizing as little as 10 ng of protein in SDS-PAGE. The mass spectroscopic analyses were carried out in the Protein Chemistry Laboratory, NCI-Frederick, Frederick, MD.

Cell Binding Assays. The human cancer cell lines SK-BR-3 (HER2 receptor-positive) and MDA-MB-468 (HER2 receptor-negative) were used to test receptor binding activity of the scFv fusion proteins. HER2 receptor expressing human breast adenocarcinoma cells (SKBR-3) and HER2 receptor negative human breast adenocarcinoma cells (MDA-MB-468) were purchased from the American type Culture Collection (ATCC, Manassas, VA). The cells were cultured in McCoy's 5A medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and penicillin/streptomycin as antibiotics at 37 °C in a humidified atmosphere containing 5% CO_2. Other culture reagents were bought from Invitrogen (Carlsbad, CA). In a typical experiment, cells were suspended in enzyme-free cell dissociation buffer (Invitrogen Corp, Carlsbad, CA) and pelleted. The cells were resuspended in phosphate buffer saline (PBS) supplemented with 5% fetal calf serum (PBS-FCS) at a concentration of 10^7 cells per mL. The samples were incubated at room temperature for 15 min to block nonspecific binding sites. The cells were washed twice with cold PBS with 1% bovine serum albumin (PBS-BSA), resuspended in PBS-FCS (10^7 cells /ml), and aliquoted in 0.1 mL samples. Various amounts (0.2, 0.5, and 1 μg) of Alexa-conjugated scFv were added to the cells, and incubations were continued for 45 min on a rotator at 4 °C in the dark. The cells were washed twice with cold PBS-BSA, suspended in PBS-BSA, and analyzed using a FACS caliber flow cytometer (Becton Dickinson, San Jose, CA), analyzing 10 000 events per sample. Herceptin was used as a positive control in these experiments to confirm HER2 expression on SK-BR-3 cells (HER2-positive) and MDA-MB-468 cells (HER2 receptor-negative).

RESULTS AND DISCUSSION

We have previously developed a novel site-specific glycoconjugation of glutathion-S-transferase, by engineering a C-terminal fusion peptide containing one Thr residue that can be glycosylated by h-ppGalNAc-T2 with a modified sugar having a chemical handle (*15*). This site-specific glycan moiety can then be conjugated with bioactive molecules carrying an orthogonal chemical group. Here, we have extended this strategy to engineer, not just one glycosylation site at its C-terminal end, but multiple sites on an antihuman HER2 receptor single-chain antibody (anti HER2 scFv) (Figure 1a). The anti HER2 scFv fusion protein is designed to have a N-terminal T7-tag, which enhances its protein expression in *E. coli* and at the C-terminal end a Tev protease cleavage site, an O-glycosylation peptide followed by a His × 6 tag (Figure 1b). For the present study,

three forms of anti HER2 scFv proteins have been constructed: the H1, H3, and H17, with C-terminal extensions having 1, 3, and 17 Thr residues in their O-glycosylation tag, respectively (Figure 1b). The two C-terminal extension peptides, H1 and H3, were designed based on the preferred acceptor peptide sequence for h-ppGalNAc-T2 enzyme, as described earlier (*15, 16*).

Expression of the Anti Her2 scFv Proteins in *E. coli* and Their *in Vitro* Folding. Although many scFv proteins have been expressed as soluble active proteins in *E. coli*, the anti HER2 scFv proteins, H1, H3, and H17, mostly formed inclusion bodies; and only less than 100 μg of soluble active protein could be obtained from 1 L of bacterial culture. Previously in our laboratory, we have used the *in vitro* *S*-sulfonation and refolding method to produce large quantities of active β-1,4-galactosyltransferase (β4Gal-T1) enzyme from inclusion bodies (*18*). Using the same method, a large quantity of a soluble form of anti HER2 scFv proteins H1, H3, and H17, with C-terminal extension, has been obtained. The scFv fusion protein has four Cys residues and has two disulfide bonds. The *in vitro* folded protein, nonreduced and reduced with β-mercaptanol, shows in SDS-PAGE a significant mobility difference between the protein bands confirming the presence of disulfide bond(s) in the soluble protein. The yield of the *in vitro* folded protein for H1, H3, and H17 proteins corresponds to 10, 10, and 2.5 mg per liter of bacterial culture. Thus, it seems that the presence of the 17 amino acid C-terminal tag does not interfere with the folding of scFv molecules. Nevertheless, the 50 amino-acid-long tag significantly reduces the folding efficiency. All the refolded proteins can be concentrated to at least 10 to 15 mg/mL concentrations, without any precipitation.

Glycosylation of H1, H3, and H17 anti-HER2 scFv Proteins. As a first step, the H1, H3, and H17 scFv fusion proteins were glycosylated overnight with h-ppGalNAc-T2, using UDP-GalNAc as the donor sugar substrate. The C-terminal fusion polypeptide from these proteins was released by Tev protease digestion and analyzed by mass spectrometry (MALDI-TOF) (Figure 3). The MS analysis of the glycosylated peptide tags from H1 and H3 proteins showed that they carry 1 and 3 GalNAc sugars, respectively (Figure 3a,b), suggesting that the H1 protein has been fully and homogenously glycosylated while the H3 proteins is mostly glycosyled with three GalNAc sugars. However, the polypeptide released from H17 scFv protein by Tev protease was glycosylated with a maximum of 11 sugars instead of 17 sugars (Figure 3c). The catalytic reaction using UDP-α-2-keto-Gal as donor substrate was carried out under the same conditions as the one used for UDP-GalNAc. After the glycosylation, a small amount of the glycoprotein was subjected to the Tev digestion, and the released C-terminal fusion glycopeptides was analyzed by MALDI-TOF analysis. The glycopeptide from the H1 protein was found fully glycosylated with a single 2-keto-Gal sugar, whereas the glycopeptide from H3 and H17 was found to be a mixture of peptides with 2 to 3 and 5 to 8 2-keto-Gal sugars, respectively (Figure 3d,e,f). These results show that all the Thr residues present in the O-glycosylation tag of the H3 and H17 scFv proteins are not homogenously glycosylated by the h-ppGalNAc-T2, particularly with the modified sugar.

In humans, the h-ppGalNAc-T2 enzyme exists as a family of 17 enzymes, namely, ppGalNAc-T1 to ppGalNAc-T17 (*19*). It is well-known that h-ppGalNAc-T2 efficiently glycosylates nonconsecutive Thr residues, while ppGalNAc-T10 uses glycopeptide as the substrate and efficiently glycosylates the consecutive Thr residues if either one is glycosylated (*16, 20*). The O-glycosylation tag of the H17 fusion protein has 10 nonconsecutive Thr residues. Thus, it is not surprising to observe that its tag is glycosylated by h-ppGalNAc-T2 with only 11 GalNAc sugars, and to fully glycosylate this tag, it may be essential to use the ppGalNAc-T10 enzyme, in addition to h-ppGalNAc-T2. Even though the 2-keto moiety in the 2-keto-Gal resembles the N-acetyl moiety in GalNAc, due to its conformational flexibility, the 2-keto-Gal sugar may not be as preferred a donor sugar substrate as is GalNAc by the h-ppGalNAc-T2 enzyme; therefore, the catalytic efficiency of h-ppGalNAc-T2 is expected to be slightly poorer for the 2-keto-Gal transfer than for GalNAc.

Preparation of Alexa488-Anti-Her2ScFv Conjugates and Their Binding to HER2-Receptor-Expressing Cells. To demonstrate a site-specific conjugation, the H1, H3, and H17 scFv proteins with 2-keto-Gal sugars were conjugated with a fluorescence probe, Alexa488, with an orthogonal chemical group, the aminoxy group. The Alexa488-conjugated H1, H3, and H17 scFv proteins can be detected on an SDS-PAGE by their fluorescence emission, even at nanogram amounts (Figure 4a). Interestingly, the H3 and H17 scFv proteins that carry more than one Alexa488 molecule are observed as sharp protein bands in the SDS-PAGE. This is in contrast to limited but random conjugated proteins, where the conjugated proteins are often observed as a smeared broad band in SDS-PAGE. For example, the streptavidin-Alexa488 conjugate protein from Invitrogen is observed as a broad band in SDS-PAGE (Figure 4b). Thus, observation of a sharp protein band on the gel indicates conjugation of the fluoroprobes to unique site(s) on the protein molecules. The observation that the fluorescence emission from H17 protein was not significantly enhanced, compared to the H1 scFv protein, may be due to self-quenching of conjugated Alexa488 molecules. It is known that multiple fluorescence probes conjugated to protein molecules show self-quenching. It is interesting to observe that even though the H3 and H17 protein-Alexa488 conjugates are heterogeneous, arising from their glycosylation with 2-keto-Gal, the protein bands in the SDP-PAGE gel remain sharp. Although the actual number of Alexa-488 molecules that are conjugated to the 2-Keto-galactosylated scFv molecules could not be determined by conventional MALDI-TOF analysis, in our previous studies it was shown that when, instead of Alexa488, biotin molecule was used for conjugation under similar reaction condition, all the 2-keto-Gal sugar molecules were completely biotinylated as determined by MALDI-TOF analysis (*14, 15*). Since we have used nearly 1000-fold excess of Alexa488 molecules in the conjugation reaction, all the 2-keto-Gal sugar molecules are expected to be conjugated with Alexa488 molecules.

Cell-binding assay by FACS methodology analysis shows that Alexa488-conjugated scFv fusion proteins H1, H3, and H17 bind specifically to cells that express HER2 receptors on the cell surface (Figure 5). We did not observe any binding of these modified scFv molecules above background levels when MDA-MB-468 cells (HER2 receptor-negative) were used (Figure 5b). In these experiments, FACS spectra using various amount of scFv fusion proteins ranging from 200 ng to 2.5 μg showed no significant difference, suggesting that even a nanogram quantity of the H1, H3, or H17 protein was sufficient for the detection of HER2 receptor. Although there is enhanced fluorescence emission (FL1-H) with H3 and H17, compared to H1, it is not linearly proportional to the number of additional fluorescence probes conjugated to these proteins (Figure 5a, insert table). This kind of observation for the multiple conjugations of protein probes to proteins is known due to the internal quenching. However, our technology is likely to yield similar conjugates with multiple numbers of MRI image contrast agents (on a single protein molecule), since there is no quenching effect in MRI. The present results suggest that the *in vitro* folded scFv is biologically active and the incorporation of the sugar and the Alexa488 moieties at the C-terminal end had no or little effect on their affinity for the HER2 receptor, thus demonstrating that

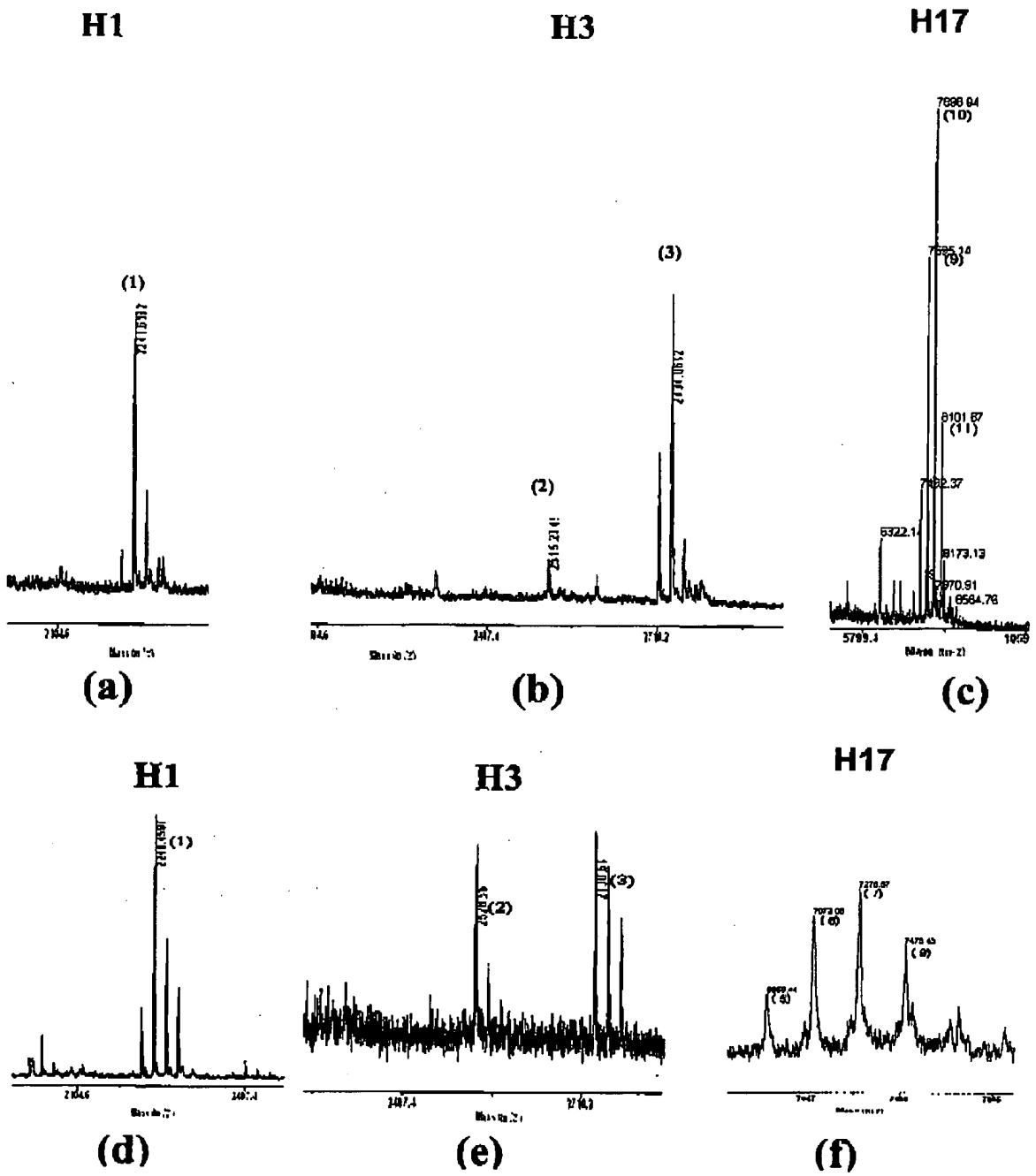

Figure 3. The mass spectrum (MALDI-TOF) of the Tev protease cleaved glycosylated polypeptides with GalNAc (a,b,c) and 2-keto-Gal (d,e,f) from H1, H3, and H17 anti HER2 scFv fusion proteins, respectively. The number of sugar molecules present in the peptide is shown in the parentheses next to the mass value of the corresponding peptide peak. The H1 protein is fully glycosylated with GalNAc sugar (a) while a small trace amount of unglycosylated peptide is observed when 2-keto-Gal was used (d). Although all three Thr residues in the fusion peptide from H3 have been mostly glycosylated with GalNAc, a small trace amount of mono- and diglycosylated forms are observed (b). However, under the same glycosylation condition, a mixture of fusion peptides with two and three 2-keto-Gal has been observed (e) for the H3 protein. Similarly, the H17 fusion protein is glycosylated to a maximum of 11 Thr residues with GalNAc sugar (c), compared to eight with 2-keto-Gal (f).

even a large fusion protein with many conjugated fluorescence probes does not affect the affinity of the scFv to its antigen.

CONCLUSION

Our current method offers a unique possibility not only for site-specific conjugation but also for multiple site conjugations of bioactive molecules at the C-terminal end of the scFv molecule through modified sugars. The proteolysis of the fusion peptide is an important factor for *in vivo* studies. The presence of glycosylation is known to hinder the proteolysis of peptides, and furthermore, the modified sugars are conjugated with bulky bioactive molecules; thus, their susceptibility to proteolysis is

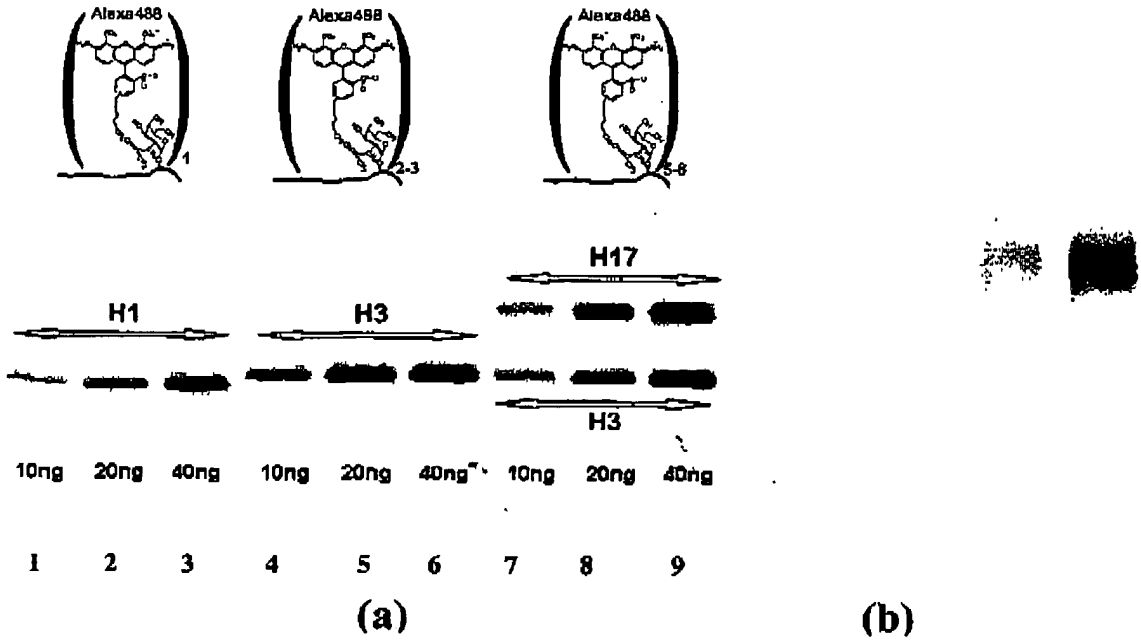

Figure 4. (a) Fluorescence emission of the Alexa488 conjugated H1, H3, and H17 anti HER2 scFv fusion proteins from the nonreduced SDS-PAGE. Lanes 1–3, 3–5, and 7–9 show each 10 ng, 20 ng, and 40 ng of H1, H3, and a mixture of H3 and H17 proteins, respectively. The H17 protein has 5 to 8 conjugated molecules of Alexa488. The fluorescence emission image of the SDS-PAGE gel was scanned using Hitachi FMBIO II fluorescence imaging system. Even 10 ng fusion proteins can be very well detected, and the protein bands are sharp. (b) Fluorescence emission from various amounts of streptavidin-Alexa488 conjugate on nonreduced SDS-PAGE. The protein conjugate from Invitrogen was prepared by a random but limited conjugation method.

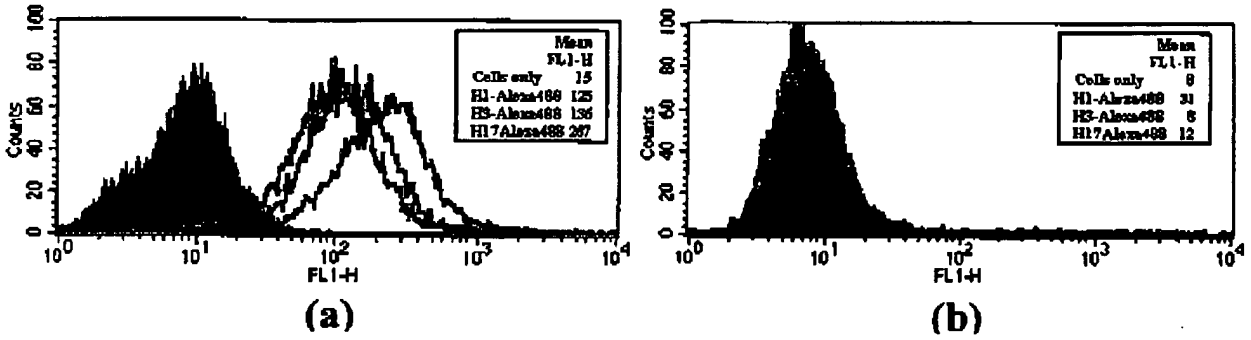

Figure 5. (a) FACS analysis on the binding of Alexa488 conjugated H1, H3, and H17 fusion proteins to SKBR3 cells. 200 ng of the each conjugated protein was used in the assay. (b) Binding of H1, H3, and H17 proteins did not occur with MDAMB-468 cells, since these cells do not express HER2 receptors. The insert table lists the mean FL1-H value for cells without and with bound antibody conjugates. Although the H17 conjugates have nearly seven more fluorescence probes, compared to H1, due to self-quenching there was no linear increase in the fluorescence emission.

expected to be much more limited compared to that of nonglycosylated fusion peptide. Similarly immunogenicity due to the fusion peptide and the modified sugars may be concern. However, it is possible that the fusion peptide may not be well exposed to the environment due to the presence of bulky conjugated bioactive molecules that may reduce the *in vivo* immunogenic effect. Such concerns are associated with most conjugated molecules that are being used or planned for use in *in vivo* studies.

Previously, scFv protein with large protein-like green fluorescence protein fused at its C-terminal end has been found to bind to the antigen without compromising its affinity (*21*). Also, a large mucin protein like, muc6, has been expressed as a soluble protein in *E. coli* and has been *in vitro* glycosylated with more than 50 sugars with GalNAc, using ppGalNAc-T1 (*22*). Therefore, using our present site-specific and multiple site conjugating method, scFv proteins with a C-terminal muc6 fusion protein can be glycosylated with modified sugars and conjugated with bioactive molecules. Such complexes are expected to carry not just a few but several tens of bioactive molecules conjugated to scFv molecules. The methodology described here can generate site-specific and multiple site-conjugated antibody-bioactive molecules that are in great need for the development of targeted MRI image contrast agents and a targeted drug delivery system.

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LITERATURE CITED

(1) Carter, P. J., and Senter, P. D. (2008) Antibody-drug conjugates for cancer therapy. *Cancer J. 14*, 154–69.
(2) Deonarain, M. P. (2008) Recombinant antibodies for cancer therapy. *Expert Opin. Biol. Ther. 8*, 1123–41.
(3) Wu, A. M., and Senter, P. D. (2005) Arming antibodies: prospects and challenges for immunoconjugates. *Nat. Biotechnol. 23*, 1137–46.
(4) Schrama, D., Reisfeld, R. A., and Becker, J. C. (2006) Antibody targeted drugs as cancer therapeutics. *Nat. Rev. Drug Discovery 5*, 147–59.
(5) Lambert, J. M. (2005) Drug-conjugated monoclonal antibodies for the treatment of cancer. *Curr. Opin. Pharmacol. 5*, 543–9.
(6) Stimmel, J. B., Merrill, B. M., Kuyper, L. F., Moxham, C. P., and Hutchins, J. T. (2000) Site-specific conjugation of serine — cysteine variant monoclonal antibodies. *J. Biol. Chem. 275*, 30445–50.
(7) Albrecht, H., Burke, P. A., Natarajan, A., Xiong, C. Y., and Kalicinsky, M. (2004) Production of soluble ScFvs with C-terminal-free thiol for site-specific conjugation or stable dimeric ScFvs on demand. *Bioconjugate Chem. 15*, 16–26.
(8) Carrico, I. S., Carlson, B. L., and Bertozzi, C. R. (2007) Introducing genetically encoded aldehydes into proteins. *Nat. Chem. Biol. 3*, 321–2.
(9) Kiick, K. L., Saxon, E., Tirrell, D. A., and Bertozzi, C. R. (2002) Incorporation of azides into recombinant proteins for chemo-selective modification by the Staudinger ligation. *Proc. Natl. Acad. Sci. U.S.A. 99*, 19–24.
(10) Davis, B. G., Lloyd, R. C., and Bryan Jones, J. (1998) Controlled site-selective glycosylation of proteins by a combined site-directed mutagenesis and chemical modification approach. *J. Org. Chem. 63*, 9614–5.
(11) Ramakrishnan, B., and Qasba, P. K. (2002) Structure-based design of beta 1,4-galactosyltransferase I (beta 4Gal-T1) with equally efficient N-acetylgalactosaminyltransferase activity: point mutation broadens beta 4Gal-T1 donor specificity. *J. Biol. Chem. 277*, 20833–9.
(12) Boeggeman, E., Ramakrishnan, B., Kilgore, C., Khidekel, N., Hsieh-Wilson, L. C., Simpson, J. T., and Qasba, P. K. (2007) Direct Identification of nonreducing GlcNAc residues on N-glycans of glycoproteins using a novel chemoenzymatic method. *Bioconjugate Chem. 18*, 806–14.
(13) Pasek, M., Ramakrishnan, B., Boeggeman, E., Manzoni, M., Waybright, T. J., and Qasba, P. K. (2009) Bioconjugation and detection of lactosamine moiety using alpha1,3-Galactosyltransferase mutants that transfer C2-modified galactose with a chemical handle. *Bioconjugate Chem. 20*, 609–18.
(14) Qasba, P. K., Boeggeman, E., and Ramakrishnan, B. (2008) Site-specific linking of biomolecules via glycan residues using glycosyltransferases. *Biotechnol. Prog. 24*, 520–6.
(15) Ramakrishnan, B., Boeggeman, E., and Qasba, P. K. (2007) Novel method for in vitro O-glycosylation of proteins: application for bioconjugation. *Bioconjugate Chem. 18*, 1912–8.
(16) Gerken, T. A., Raman, J., Fritz, T. A., and Jamison, O. (2006) Identification of common and unique peptide substrate preferences for the UDP-GalNAc:polypeptide alpha-N-acetylgalactosaminyltransferases T1 and T2 derived from oriented random peptide substrates. *J. Biol. Chem. 281*, 32403–32416.
(17) Hubé, F., Mutawe, M., Leygue, E., and Myal, Y. (2004) Human small breast epithelial mucin: the promise of a new breast tumor biomarker. *DNA Cell Biol. 23*, 842–9.
(18) Boeggeman, E. E., Ramakrishnan, B., and Qasba, P. K. (2003) The N-terminal stem region of bovine and human beta1,4-galactosyltransferase I increases the in vitro folding efficiency of their catalytic domain from inclusion bodies. *Protein Expr. Purif. 30*, 219–29.
(19) Ten Hagen, K. G., Fritz, T. A., and Tabak, L. A. (2003) All in the family: the UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferases. *Glycobiology 13*, 1R–16R.
(20) Kubota, T., Shiba, T., Sugioka, S., Furukawa, S., Sawaki, H., Kato, R., Wakatsuki, S., and Narimatsu, H. (2006) Structural basis of carbohydrate transfer activity by human UDP-GalNAc: polypeptide alpha-N-acetylgalactosaminyltransferase (pp-GalNAc-T10). *J. Mol. Biol. 359*, 708–27.
(21) Peipp, M., Saul, D., Barbin, K., Bruenke, J., Zunino, S. J., Niederweis, M., and Fey, G. H. (2004) Efficient eukaryotic expression of fluorescent scFv fusion proteins directed against CD antigens for FACS applications. *J. Immunol. Methods 285*, 265–80.
(22) Freire, T., Lo-Man, R., Piller, F., Piller, V., Leclerc, C., and Bay, S. (2006) Enzymatic large-scale synthesis of MUC6-Tn glycoconjugates for antitumor vaccination. *Glycobiology 16*, 390–401.

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